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der Vetsuisse-Fakultät Universität Zürich

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Elucidation of the interaction network between yeast DNA processing proteins using endogenously tagged ORFs and the LiquiChip technology

INAUGURAL-DISSERTATION

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This work is dedicated to my brother

René Hort

who taught me how to become

a great warrior

Figures and Tables

1 Summary

In the yeast *Saccharomyces cerevisiae*, we wanted to set a foundation for examination of protein interactions involved in DNA replication, recombination and repair. The goal was to establish a novel *in vivo* high-throughput technique to test for binary protein interactions in yeast. For this reason, we created two new yeast arrays by endogenously tagging the selected yeast ORFs with 6xHis (bait) and 3xVSV (prey) tags, respectively. In total, we generated 927 tagged ORFs involved in DNA processing, of which 470 are baits and 457 preys. Using an array format, haploid yeast were mated to generate diploids expressing both bait and prey proteins. Then, protein extracts were prepared and incubated with Ni-Beads, binding to the bait and a fluorescently labeled antibody recognizing the prey. We analysed the samples by the LiquiChip technology, originally developed at Qiagen Inc., which uses lasers to examine single beads loaded with bait for the presence or absence of the fluorescently labeled prey protein, thus determining the interaction status between bait and prey. As a proof of principle, we examined control strains of the Casein Kinase II complex. Using this approach, we were able to detect interactions between different members of this Protein Kinase II family. To verify the binding of the bait to the beads, as well as the presence of the interacting partner, we performed *in vivo* pull down assays using the nickelbeads following western blot analyses in order to detect the interacting prey protein.

2 Abbreviations

AD	activation domain
Amp	ampicillin
BD	binding domain
bp	base pair(s)
BSA	bovine serum albumine
cDNA	control DNA
CIP	calf intestinal phosphate
dATP	desoxyadenosin-5'-triphosphate
dCTP	desoxycytidin-5'-triphosphate
ddH2O	double distilled water
dGTP	desoxyguanosin-5'-triphosphate
dH2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	nucleotides
ds	double-stranded
dTTP	desoxythymidin-5'-triphosphate
ECL	enhanced chemiluminescence
E.coli	*Escherichia coli*
FECL	fento enhanced chemiluminescence
His	histidine
IgG	immuno globulin G
Kan^r	kanamycin resistancy
kDa	kilodalton
Leu	leucine
LiAc	lithium acetate
Lys	lysine
MbYTH	split-ubiquitin membrane yeast two-hybrid sytem
Met	methionine
MFI	mean fluorescence intensity
mRNA	messenger RNA
OD	optical density

o/n	over night
PBS	phosphate buffered saline buffer
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethane sulfonylfluorid
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
Rxn	reaction
S.cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
ss	single-stranded
TBS	tris-buffered saline
TBST	tris-buffered saline containing Tween 20
Ura	uracil
VSV	vesicular stomatitis virus
YTH	yeast two-hybrid

3 Introduction

3.1 Proteomics: communication of living cells

Up to now classical genetics and biochemistry were the main disciplines used for the investigation of an organism concerning its development, reproduction, behavior and ageing. However, the availability of the complete genome sequence for the *Homo sapiens* (Venter *et al*., 2001) and many other model organisms including *Mus musculus* (mouse) (Waterston *et al*., 2002), *Arabidopsis thaliana* (plant) (Arabidopsis Genome Initiative, 2000), *Drosophila melanogaster* (fruit fly) (Giot *et al*., 2003), *Caenorhabditis elegans* (worm) (Walhout *et al*., 2000) as well as *Saccharomyces cerevisiae* (baker's yeast) (Uetz *et al*., 2000; Ito *et al*., 2001) has changed the approach of biological investigation. Since the first complete genome of a free-living organism, *Haemophilus influenzae* (bacterium) was sequenced in 1995 (Fleischmann *et al*., 1995) an overwhelming amount of genetic data has been produced. The new challenge to the scientific community today is the determination of the function of thousands of uncharacterized genes, 92% of all human genes have no defined function, and thus apply this information to characterize the proteins, their interacting partners and their role in the cellular processes.

The genome sequence alone cannot fully describe the intricate working of a genetic system for one gene can encode for several proteins in a process called alternative splicing. In addition, gene products, proteins, are much more complicated than genes. Unlike genes, proteins get modified after their translation: they acquire phosphate groups (phosphorylation), methyl groups (methylation), acetyl groups (acetylation) or disulfide-bridges (oxidation). They get shortened in their sequence by aminopeptidases or spliced into different smaller proteins. They convert into a glycoprotein by acquiring sugar chains and they bind to coenzymes and prosthetic groups to reach their final functionality. It is the proteins that execute the genetic program, and those that are actually produced by a cell at any given time constitute its "proteome" (Kahn, 1995). Unlike the genome, the proteome is very dynamic. The expressed proteins depend on the stage of the cell cycle, the development of the organism as well as on the external stimuli. In addition, the proteins form large interaction networks in order to communicate, regulate and support each other.

An emerging field of research, which develops techniques to study the entire complement of proteins within the genome, is termed "proteomics" (Banks *et al*., 2000). The goal of proteomics is to analyze each protein's expression and function. This can be achieved by determining when and where the proteome of an organism is expressed, identifying posttranslational modifications that regulate the protein's function, determining the protein's localization within the cell as well as by comparing protein profiles of cells, tissues and organs. However, to fully understand the cellular machinery, simply listing the proteins is still not enough. All the interactions between the proteins need to be delineated as well, for protein interactions play a role in basically all events taking place in a cell (Von Mering *et al*., 2002) causing the characterization of physical protein-protein interactions, so called "interactive proteomics", to be recognized as a key objective in proteomics research (Auerbach *et al*., 2002). Thus, since most of the cellular processes are regulated by multiprotein complexes, the disturbances of protein-protein interactions within a cell can lead to many diseases. Therefore, an important task of proteomics is to elucidate the interactions between individual members of the proteome in the normal and diseased state (Phizicky *et al*., 2003) helping to gain better insight into the pathway of illnesses and giving way for new therapeutical approaches.

3.2 Yeast: a model system in proteomics

The genome of the baker's yeast *Saccharomyces cerevisiae* was the first eukaryotic genome to be completely sequenced. Its sequence of 12,068 kilobases defines 5885 potential protein encoding genes as well as information about the higher order organization of yeast's 16 chromosomes and allows some insight to their evolutionary history (Goffeau *et al*., 1996). Since many yeast cells have homologues in higher eukaryotic systems, approximately 70% of its genes are similar to the ones in the human genome, it can be used as a model for the eukaryotic genome. In addition to this *S. cerevisiae* has many intrinsic advantages for an experimental system, which make it almost as easy to work with as bacteria: it is a unicellular organism and, therefore, gene functions can be detected easier. Its quick growth allows thousand of clonal yeast colonies to be cultured within two days. The genome is small which simplifies both, genetic and molecular analysis and allows a genomic library to be carried on a few thousand plasmids. Furthermore, yeast can be grown

on defined media, which gives complete control over its chemical and physical environment, and its life cycle is ideally suited to classical genetic analysis. One of the biggest advantages, however, is the budding of yeast. It can either be maintained as haploid, as to say a- or α-strain, which contain one genome complement, or as diploid, the mating of the a- and α-strain, which, therefore, consist of two genome complements. So, genetically recessive mutants can be easily obtained by working with haploid cells. Furthermore, genetic complementation, the geneticist's fundamental tool, can be done simply by mating two different haploid mutants to form a diploid (Watson *et al*., 1992). Apart from this, transformed DNA is efficiently integrated by homologous recombination in this organism, thus facilitating gene cloning and reverse genetics (Forsburg, 2001; Stagljar, 2002).

Even though these facts make baker's yeast a highly versatile unicellular eukaryotic system to study basic cellular processes, the task still remains to elucidate the biological functions of all its genes. At present, eight years after the completion of the yeast genome, approximately 3800 yeast genes have been characterized by genetic or biochemical methods and an additional 600 have been determined based on homologues in other organism. This reveals that there still exist about 1800 genes, which encode proteins of unknown functions, highlighting the great disparity between sequencing and functional analysis. In other words, an important challenge of the "post-genomic era" is to develop strategies that use complete genome sequences to accelerate functional predictions for the gene products (Kumar and Snyder, 2001; Stagljar, 2002). To answer this challenge, researchers have developed different high-throughput strategies to help them understand the function of each gene in the genome. Projects that are already underway include DNA microarray technology to analyze the expression profiles of genes, the analysis of mRNA expression patterns using in situ hybridization, loss-of-function approaches combined with subcellular localization screens, the large-scale localization of proteins within cells, *in silico* methods for the determination of protein fusions, gene neighboring and structural predictions, the systematic isolation of cDNA sequences across the entire genome, as well as the use of high-throughput assays to isolate interacting protein pairs (Auerbach *et al*., 2002). The success of this work, however, largely depends on the availability of easily applicable genetic and biochemical methods to manipulate yeast strains (Knop *et al*., 1999).

3.3 Proteomics technologies

The examination of physical protein-protein interactions can be divided into biochemical and genetic methods. Biochemical approaches, such as co-immunoprecipitaion and co-purification through chromatographic columns, is often difficult and time consuming since it requires harsh treatment for cell disruption and a unique optimization for each protein complex. However, its popularity increased due to the rapid advances of protein analytical technologies, including mass spectrometry and sequence databases. Thus making mass spectrometry-based interactive proteomics the method of choice for analyzing functional protein complexes (Aebersold and Mann, 2003).

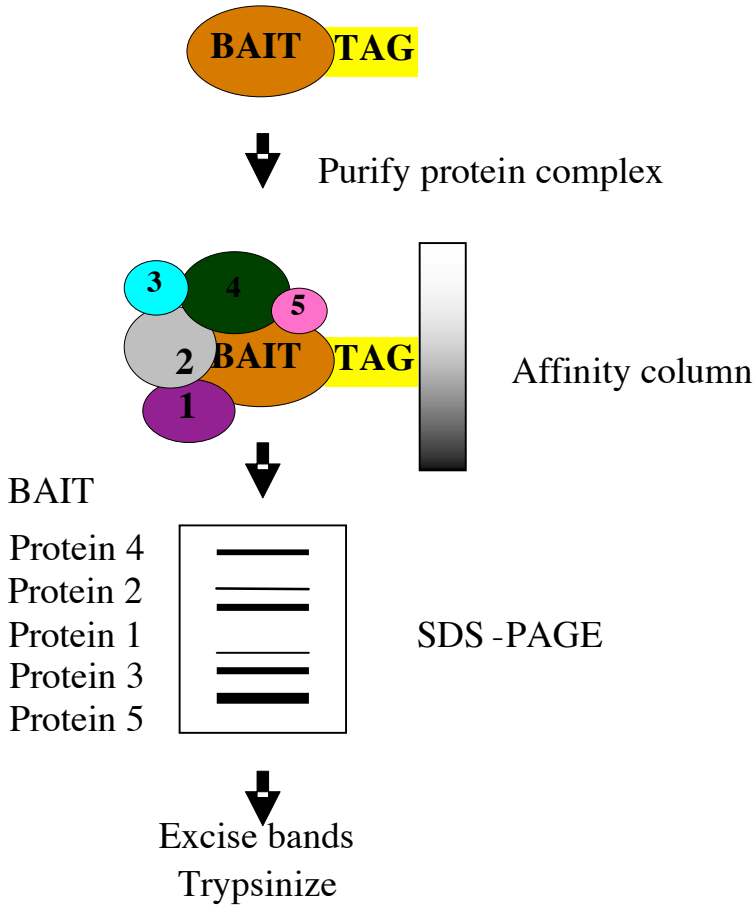

Figure 1. Mass spectrometry. After labeling the bait with a tag, the protein complex, encompassing the bait, gets purified using an affinity column. Thus, by performing an SDS-PAGE, the interacting partners of the bait can be detected and sequenced.

Along these lines, systematic protein complex studies exist using affinity-tagged proteins expressed in yeast as baits to capture and identify their associated proteins (Gavin *et al.*, 2002; Ho *et al.*, 2002). Other new strategies include the isotope labeling by amino acids in cell culture (SILAC) (Ong *et al.*, 2002) and the isotope-coded affinity tag (ICAT) (Gygi *et al.*, 1999). The genetic systems, which are based on the detection of protein-protein interactions *in vivo*, have quickly become a popular tool due to their minor need for individual optimization and their ease for screenings in a high-throughput format (Vidal and Legrain, 1999). The yeast two-hybrid system (YTH), the most common method, can be used to screen for binary interactions between proteins in the cell nucleus (Fields, 1989).

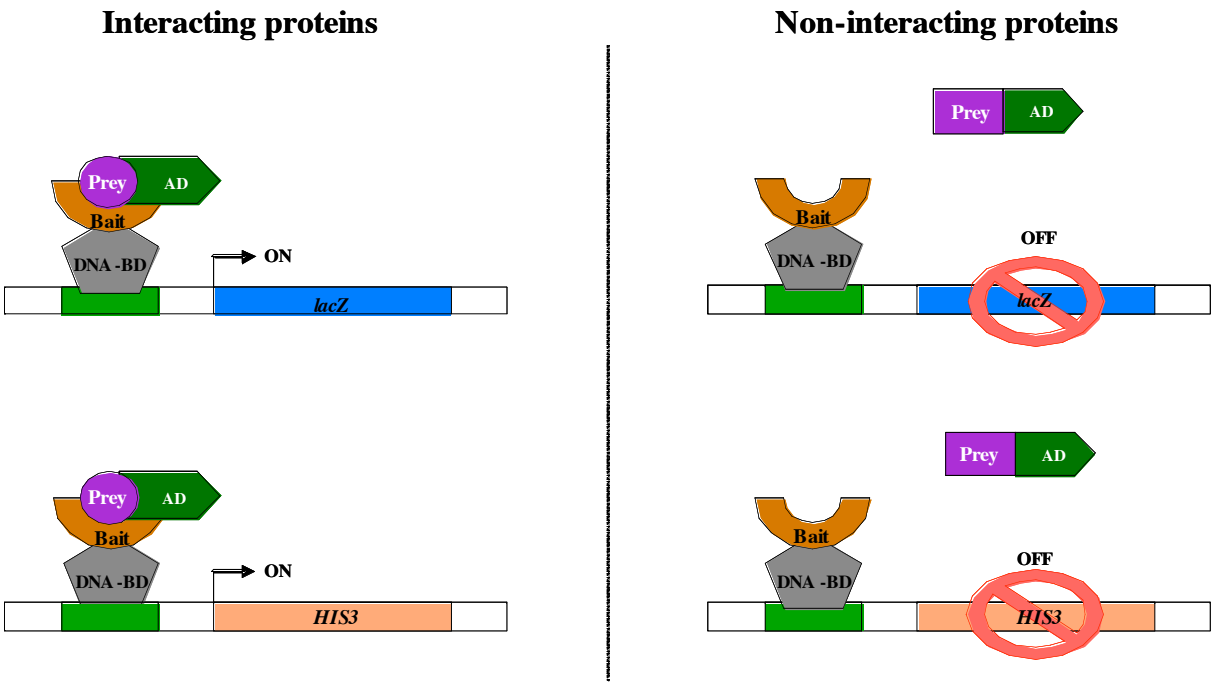

Figure 2. Principles of the yeast two-hybrid system (YTH). Many eukaryotic transcriptional activators consist of two physically separable and modular domains (Ma and Ptashne, 1987), a DNA-binding domain (BD) and a transcription activation domain (AD). The DNA-binding domain serves to target the transcription factor to specific promoter sequences. The activation domain recruits the RNA polymerase II complex to the promoter site leading to transcription of downstream genes. Since both domains are required, transcription only takes place if the two protein of interest interact with each other.

With this method many novel components of the cell signaling pathways have been found (Fashena *et al*., 2000) and genome-wide YTH projects have been conducted to analyze protein-protein interactions at a global level in different cellular organism, including yeast (Uetz *et al*., 2000; Ito *et al*., 2001), producing a wealth of new protein interaction data. The major disadvantage of the YTH is that the interaction can only occur in the nucleus. Consequently, transmembrane proteins cannot be efficiently studied by this method.

However, an alternative YTH approach exists to study membrane proteins, the so called split-ubiquitin membrane yeast two-hybrid system (MbYTH) (Stagljar *et al*., 1998; Thaminy *et al*., 2003; Iyer *et al*., 2005). MbYTH uses the split-ubiquitin approach in which the reconstitution of two ubiquitin halves is mediated by a specific protein-protein interaction (Johnsson and Varshavsky, 1994). The interaction between two membrane proteins results in ubiquitin reconstitution and leads to the proteolytic cleavage and subsequent release of a transcription factor that triggers the activation of a yeast reporter gene system, enabling indirect detection of a protein interaction (Stagljar, 2003). Even thought the sensitivity of this method is very high, it lacks on selectivity leading to a high amount of false positive, presumable due to protein interactions that normally do not exist *in vivo*. Furthermore, neither the MbYTH nor the traditional YTH can be applied to study protein complexes, but can only detect binary interactions.

The future progress in the field of proteomics largely depends on the refinement of the existing methods, their applicability in a high-throughput manner as well as in the creation of new methods undertaking different approaches. To create a valid data set the limitation of each technology has to be taken into account and complemented by other additional techniques. Thus, the best approach to identify protein-protein interaction partners is likely to be a combinatorial one (Stagljar, 2003).

3.4 Creating a new method in the field of proteomics

The goal of the project was to create a new method in the field of proteomics fulfilling the following criteria: easy handling to save time and material, giving way for high-throughput assays. A broad application range enabling the examination of many types of protein-protein interactions and the possibility to expand the method in other directions. An *in vivo* testing, reassuring the results to be as reliable as possible. A high selectivity and specifity leading to a minimum of false positive or negative results and enabling the detection of weak interactions. This was achieved by combining a genetic method together with a biochemical one, as to say by endogenously tagging yeast ORFs and testing the possible interaction partners via the LiquiChip technology, which enabled us to determine the interaction status between two proteins, a bait and prey by using a bead-based platform. The bait protein was fused with a tag and the prey to an epitope marker. To this protein extract fluorescently labeled beads, binding to the tag of the bait protein, and a fluorescently labeled antibody directed towards the tag of the prey were added and then analyzed by the machine.

The LiquiChip uses two lasers, one for the detection of the beads and the other for the antibody (Figure 3). Since only a single file passage of beads is possible, each bead can be irradiated by both lasers at the same time so that the fluorescent signals generated from the beads and the reporter molecules are simultaneously recorded. Since the fluorescent antibody signal is only recorded in conjunction with the bead fluorescence, signals from free, unbound fluorescently labeled antibodies are not recorded. Therefore, simultaneous detection of green and red fluorescence constitutes a positive interaction between two proteins. The only disadvantage of the LiquiChip system is that it is only able to detect binary interactions. However, it has several advantages, which may compliment existing biochemical techniques. Since no washing steps are required to remove unbound reporter, there is less chance of disrupting weak interactions. The use of the laser technology provides an approximately 100 times more sensitive detection method compared to a conventional immunoassay (Qiagen). Furthermore, the low requirement of working steps included, as well as the possibility to test up to 96 different samples at once, enable high-throughput assays.

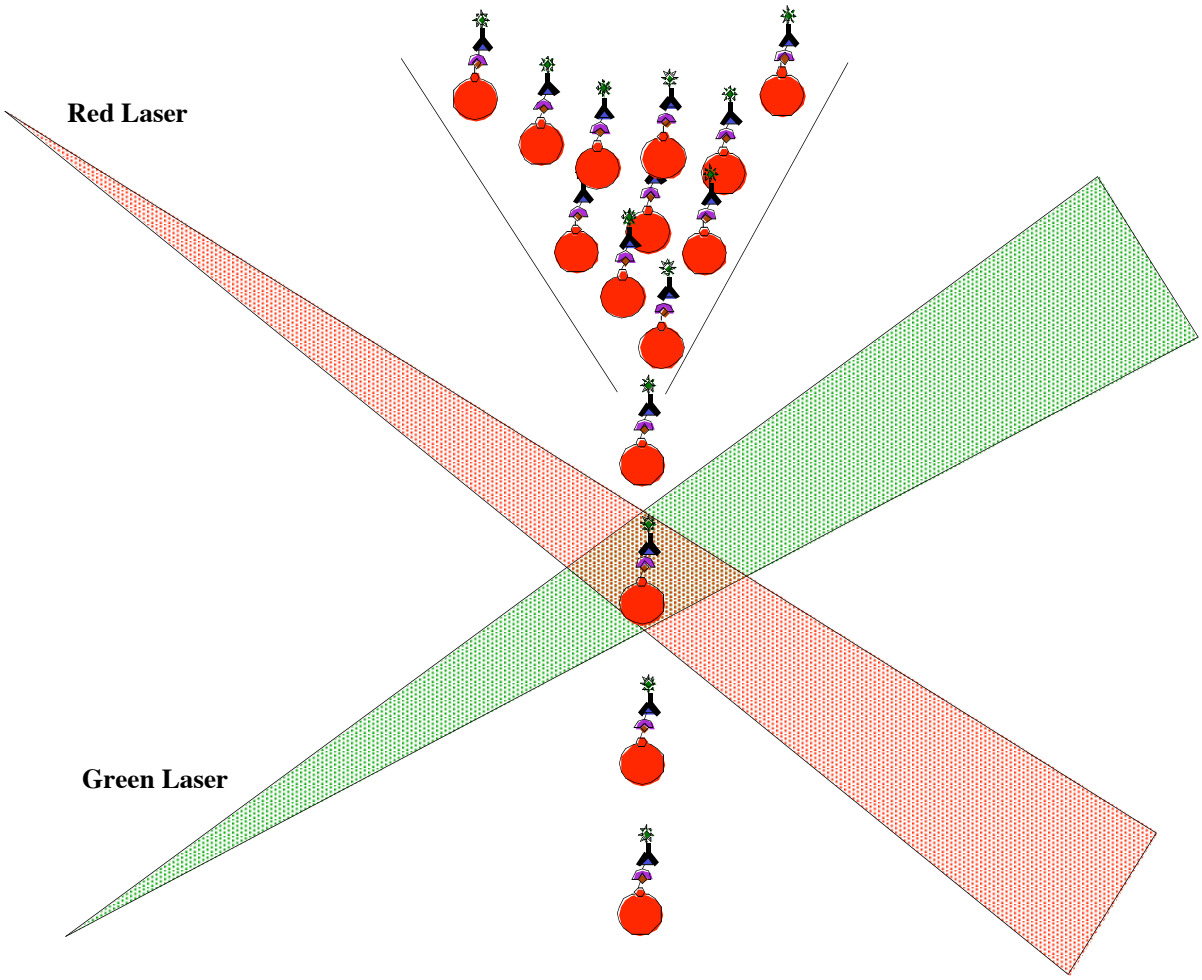

Figure 3. The principle of the LiquiChip technology. The fluorescently labeled beads bind to the tag of the bait (Protein 1). The prey (Protein 2) is marked by a fluorescently labeled antibody. If the two proteins of interest interact with each other, the prey is also attached to the bead through the bait. Therefore, both lasers (red: recording the fluorescenting signal of the beads, green: detecting the signal of the antibody) get a signal simultaneously as a prove of protein interaction.

In addition to this, the genetic approach, by endogenously tagging of the proteins, makes the *in vivo* testing of the proteins possible and provides not only a platform to build up other techniques in the field of proteomics, but also reassures that the expression of the proteins is carried out from their own promoters. Therefore, the normal levels of the tagged proteins will be expressed and should maintain the proper stoichiometry with respect to other cellular proteins.

By creating two differently tagged libraries this work will be available for the entire yeast community giving the possibility for a wide variety of array-based manipulations such as conventional pull down assays and co-localization studies.

3.5 Proteins involved in cell replication, repair and recombination

In order to survive and keep up the proper cell function each cell is faced with the crucial task of maintaining the stability of its genome. Not only does this task belong to the proteins involved in DNA repair, checking its integrity randomly, but also to proteins assuring the correct replication of the entire DNA in the case of mitosis or recombination for meiosis.

These fundamental and complicated events are carried out by a pathway of protein interactions. The loss of one protein function can, therefore, have a severe impact on several enzymatic cascades changing not only the cells behavior but also influencing the integrity of a cell community. Despite the expanse in evolutionary time, the functions of these proteins are strikingly similar in yeast compared to higher organism. Therefore, with the search for new interacting partners, a deeper insight into the signaling cascade in the field of cell replication, repair and recombination should be obtained inclining that the knowledge of their exact working pattern can predict human gene functions and illuminate the pathway of cell based diseases.

A second focus was set on uncharacterised proteins, since the method enables one to investigate their biological function. This can be obtained by confirming their interaction with other, already known, proteins: if the function of one protein is known, then the function of its binding partner is likely to be related. This concept has been termed "guilt by association". When used on a large scale, it allows one to quickly assign functions to uncharacterized binding partners using a relatively small number of functionally characterized proteins (Auerbach *et al*., 2002).

4 Problem and Approach

The project can be separated into three distinct steps: the generation of an endogenously tagged yeast bait and prey DNA library. The mating of the strains in order to generate diploids, expressing both the bait and prey proteins, followed by the preparation of the protein extracts, and finally the use and concept of the LiquiChip technology to test for protein interactions between bait and prey proteins.

4.1 Generation of endogenously tagged yeast bait and prey libraries

4.1.1 Selection of the ORF

The Functional Catalogue of MIPS (http://mips.gsf.de/) served as a means to choose all the proteins involved in replication, repair and recombination in the *Saccharomyces cerevisiae*. In addition to these 209 ORFs, proteins of unknown function (Tong *et al*., 2001) as well as homologues of human disease-associated genes (Foury, 1997) were of interest. All of the chosen genes were confirmed for their function and their interacting partners by consulting the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/).

4.1.2 Digital support

To keep an overview of the data created, two different charts were established: one containing all the genes selected with their fact file and the second showing the immediate workstep on each ORF (for more details see Table 11, page 68).

4.1.3 Creation of plasmids for endogenous tagging

To generate endogenously tagged bait and prey protein open reading frames (ORFs), two different plasmids had to be generated (De Antoni and Gallwitz, 2000), which were used for the amplification of the needed PCR products. One plasmid served to tag the chosen genes used as baits, the other plasmid for the tagging of the preys.

The plasmids were needed to have two distinct tools: a resistance marker to enable only those yeast strains, which had inserted the PCR product into their genome, to grow on selected media. Also, both plasmids contained two common primer binding sites, one in front and one after the tag, in order that only one set of primers was needed for the generation of a bait and/or prey tagged ORF.

Originally three different plasmids were created to serve for the prey strain, which differed in their aminoacid composition of the tag. After the generation of six different ORFs of each tag, the plasmid was chosen to serve for the tagging of the preys that gave the best signal from performed western blots.

4.1.4 Correct insertion of the PCR fragment

To confirm that the PCR tagging cassette integrated into the proper place in the genome, a control PCR was performed. To accomplish this, a PCR reaction was made after each tagging using an ORF specific forward primer and a reverse primer, which annealed to a common binding site at the end of the tag. The only way that this particular PCR reaction could produce a fragment of the expected size was if the PCR tagging cassette inserted behind the gene to be tagged.

In addition, the colony PCR products of all the strains were sequenced over the ORF/tag junction to exclude any false results.

4.1.5 Expression of the tagged protein in the yeast line

Each manipulation on a gene could influence the protein's expression. Even though the chances of a change of expression were lessened by using short tags, the strains chosen for the LiquiChip were tested for their expression level by performing western blots.

4.2 Mating of the strains and preparation of the protein extracts

4.2.1 Mating of the strains

In order to create one strain containing both, the tagged bait and prey, two different libraries had to be created first. The bait was inserted into the haploid a-strain while the haploid α-strain served for the prey line. Those two haploid mating types of yeast could then be mated so that a diploid form containing $a \times \alpha$ chromosomes was created. To select only those colonies that were diploids, both strains were lacking a different enzyme that produced an essential aminoacid. When diploids were formed, the essential enzyme was substituted by the other strain.

4.2.2 Preparation of the protein extracts

Since yeast cells have a complex proteinaceous cell wall, which often makes lysis difficult, two types of extraction methods were tested for their feasibility and compatibility with the LiquiChip system. The first method used the mechanical action of small glass beads to shear the cell wall while the second relied on a commercially available chemical to break the cell wall and thus released the soluble proteins. Tests comparing the amount of extracted protein as well as the maintenance of protein interactions, while working with the lysates on the LiquiChip machine, were made to choose the more appropriate protein extraction method.

4.2.3 Use and concept of the LiquiChip technology

In order to get familiar with the handling of the LiquiChip technology as well as adapting its set up to yeast assays, the first tests were preformed with only purified non-yeast proteins.

4.2.4 Reducing the background

The cell lysate of the yeast was lowering the fluorescent signals dramatically. By changing buffer conditions, incubation times, as well as comparing the different protein extraction methods, the background could be reduced to a minimum.

4.2.5 Choosing the beads

Fluorescently labeled beads, which bound to the tag of the bait protein, were added to the protein extracts. Two differently coated beads were considered, both having distinct advantages: Ni-NTA-Beads, for their high binding capacity, and P-Beads for their high level of specifity towards the bait tag. To select for the proper beads, competitive assays, as well as pull downs, were made to see the reliable binding of the baits to the beads, and their fluorescent signal was compared when working with the LiquiChip technology.

4.2.6 Handling of the antibodies

Due to the fact that a fluorescent antibody directed towards our prey tag did not exist, two antibodies had to be used: the first antibody bound to the tag of the prey while the second fluorescently labeled antibody was directed towards the first one.

A profound set of comparative assays led to the accurate amount of primary and secondary antibody needed as well as to the best incubation time.

5 Materials and Methods

5.1 Materials

5.1.1 Strains

***E.coli*:** **DH5alpha**

All bacteria were grown in LB+Amp (100µg/ml Ampicillin) at 37°C

***S.cerevisiae*:** **BY4742** (MATα ura30 leu20 his31 lys20)

BY4741 (MATa ura30 leu20 his31 met150)

(Brachmann *et al*., 1998)

5.1.2 Media and Solutions

LB medium:

10 g Bacto Tryptone
5 g Bacto Yeast Extract (1%)
5 g NaCl

- Addition of dH2O to Vtot= 1 L
- Autoclaved at 121°C for 15'
- Stored at room temperature

YPD medium:

10 g Bacto Yeast Extract (1%)
20 g Bacto Peptone (2%)
20 g Glucose (2%, monohydr. or anhydr.)

- Addition of dH_2O to Vtot= 1 L
- Autoclaved at 121°C for 15'
- Stored at room temperature

Ampicillin drop-out plates:

100 ml LB medium
2 g Bacto Agar
100 µl Ampicillin (100mg/ml)

- Addition of dH_2O to Vtot= 1 L
- Autoclaved at 121°C for 15'

G418 drop-out plates:

10 g Bacto Yeast Extract (1%)
20 g Bacto Peptone (2%)
20 g Glucose (2%, monohydr. or anhydr.)
20 g Bacto Agar (2%)

- Addition of dH_2O to Vtot= 1 L
- Autoclaved at 121°C for 15'

2 ml G418 (100mg/ml)

- Poured into plates
- Stored at 4°C

-Met/-Lys drop-out plates:

1 g	Drop out mix
6.7 g	Bacto Yeast Nitrogen Base (w/o AA)
20 g	Glucose (2%, monohydr. or anhydr.)
20 g	Bacto Agar (2%)

- Addition of dH_2O to Vtot= 1 L
- Autoclaved at 121°C for 15'
- Poured into plates
- Stored at 4°C
- Poured into plates

Transfer buffer:

3.03 g	Tris
14.4 g	Glycine

- Addition of dH_2O to Vtot= 1 L
- Stored at 4°C

Added prior to use:

200 ml	Methanol

10x Loading buffer:

0.25 %	bromphenol blue
0.25 %	Xylene cyanol FF
30 %	glycerol

10x Running buffer:

60 g	Tris
300 g	Glycine
20 g	SDS

- Addition of dH_2O to Vtot= 1 L

Blocking buffer 1:

2.5 g	Milk powder (5%)
50 ml	TBS solution

Breaking buffer:

5 ml	KCL (2M)
4 ml	Hepes (500mM)
2 ml	Magnesiumacetate (100mM)
89 ml	ddH2O

Incubation buffer:

120 mM	NaCl
50 mm	Tris HCl (pH=7.5)
0.1 %	NP-40
5 mM	2-Glycerophosphate
2 mM	Magnesium acetate
10 mM	Imidazol

Added protease inhibitors before use:

2 mM	PMSF
1 μg/ml	B, P, L

20xTBS solution:

160 g	NaCl
4 g	KCl
60 g	Tris

- Addition of dH2O to Vtot= 1 L
- Adjusted to pH 7.4

TBST solution:	50 ml	20×TBS
	500 µl	Tween20 (0.05%)

- Addition of ddH2O to Vtot= 1 L
- Stored at room temperature

Solution B:	36.33 g	Tris-HCL (1.5mol/l, pH 8.8)
	0.80 g	SDS

- Addition of ddH2O to Vtot= 200ml
- Adjusted to pH 8.8
- Filtered 0.45µm

Solution C:	12.11 g	Tris-HCL (0.5mol/l, pH 6.8)
	0.80 g	SDS
	tipfull	Bromphenol blue

5.1.3 Plasmids

pYes2.1./V5-His/lacZ:	8.9 kb integrative yeast plasmid containing a GAL1 promoter, 2µ origin, Ampicillin resistance gene, URA3, V5 and 6His tag (Invitrogen)
pU6H3VSV:	(De Antoni and Gallwitz, 2000)
pU6H2MYC:	(De Antoni and Gallwitz, 2000)

pU6H2HA:	(De Antoni and Gallwitz, 2000)

5.1.4 Antibodies

LiquiChip

V5:	Rabbit polyclonal antibody directed against the V5-epitope (abcam)
VSV:	Rabbit polyclonal unconjugated antibody raised against the VSV-G-epitope (Bethyl Laboratories)
Alexa Fluor 532:	Goat anti-rabbit IgG (Molecular Probes)

Western Blot

V5:	Mouse monoclonal IgG2a antibody directed against the V5-epitope (Invitrogen)
VSV:	Mouse monoclonal antibody (Roche)
Anti-mouse IgG:	Horseradish peroxidase-conjugated linked whole antibody from sheep (Amersham)

5.1.5 Others

Drop out mix:

0.5 g Adenine
2.0 g Alanine
2.0 g Arginine
2.0 g Asparagine
2.0 g Aspartic acid
2.0 g Cysteine
2.0 g Glutamine
2.0 g Glutamic acid
2.0 g Glycine
2.0 g Inositol
2.0 g Isoleucine
0.2 g para-Aminobenzoic acid
2.0 g Phenylalanine
2.0 g Proline
2.0 g Serine
2.0 g Threonine
2.0 g Tyrosine
2.0 g Uracil
2.0 g Valine

20% SDS:

200 g SDS

- Dissolved in 1000ml dH2O

G418:

1.25 g G418
10 ml ddH2O

BSA/PBS: 0.1 g Bovine Serum Albumin

- Dissolved in 100ml PBS

10mM dNTP's:
10 µl 100mM dATP
10 µl 100mM dGTP
10 µl 100mM dCTP
10 µl 100mM dTTP
60 µl ddH2O

- Mixed thoroughly
- Stored at 4°C

10%APS: 1 g Ammonium peroxidsulfate

- Dissolved in 10ml dH2O

TEMED: Tetramethylethylenediamine

ECL: Enhanced chemiluminescent substrate for detection of HRP (PIERCE)

FECL: SuperSignal West Femto Maximum Sensitivity Substrate (PIERCE)

LiAcetate 1M:	20.2 g	LiAcetate
		- Dissolved in 100ml dH_2O
50% PEG-4000:	50 g	PEG-4000
		- Added dH_2O to Vtot= 50ml
Glass beads:	425-600	microns
Y-PER:		Yeast protein extraction reagent (PIERCE)
Primers:		Diluted in ddH_2O for a working concentration of 20pmol/µl (QIAGEN)

5.1.6 LiquiChip

Ni-NTA-Beads:	2.5×10 E 6 beads/ml, phosphate buffer containing NaCl, pH 7.4 (QIAGEN)
Penta-Beads:	α-5His-antibody (QIAGEN)

5.1.7 Pull downs

Ni-Beads: Probond™ Nickel-Chelating Resin beads, Cat no. R 801-01 (Invitrogen)

5.2 Methods

5.2.1 Amplification of pU6H3HA, -2MYC, -3VSV + pYes2.1/V5-His/LacZ

5μl (approx. 100ng/μl) of the plasmids were mixed with 50μl of competent *E.coli* cells. The mix was put on ice for 10', heat shocked for 2' at 42°C and then again put on ice for 10'. After adding 1ml of LB medium the cells were put at 37°C for 25'. Then, 900μl of the mix was added to 50ml LB medium, containing 50μl Ampicillin (100mg/ml), and incubated at 37°C o/n with shaking. To ensure the transformation was successful, 100μl of the transformed cells were plated on a LB-Ampicillin plate and incubated at 37°C. In order to purify the DNA, a Midi-Prep was performed with the QIAGEN Plasmid Purification Kit (Handbook 2000). The DNA pellet was air-dried for 1' and then resuspended in 1ml of 10mM Tris-HCL pH 8.5 or ddH2O. The determination of the yield was made with the spectrophotometer at λ 260nm (ds DNA: 50μg/ml × Dilution [5μl sample + 995μl dH20] × O.D. @ 260).

5.2.2 Construction of the bait vector

The V5-6His fragment of the pYes2.1/V5-His/LacZ was introduced into the pU6H2MYC vector (De Antoni and Gallwitz, 2000) by performing PCR on the pYES2.1/V5-His/lacZ plasmid with a 5'primer, which generated a BamH1 site and a 3'primer, which generated a Sal1 site. This fragment was cloned into the pU6H vector, which was digested with BamH1/Sal1. The resulting vector, pUV56His, contained the 6His tag flanked by the V5 epitope and the kanamycin resistance (Kan^r) gene. The Kan^r could be used as a selective marker; the yeast having integrated the cassette into their genome could be grown on G418. The V5 epitope was introduced to serve as a common primer site for PCR for bait and prey strains and could be used as a common epitope marker.

5.2.2.1 Digestion of the pU6H2MYC with BamH1 and Sal1

First, the pU6H2MYC plasmid had to be digested in order to integrate the V5-6His fragment. To 5μl of pU6H2MYC 37.5μl ddH2O, 5μl Unique BamH1 buffer and 1.5μl of BamH1 and Sal1 (20U/μl) was added. After a short spin in the centrifuge (13.5 rpm) the mixture was incubated at 37°C for 1.5 hours. Treatment with Calf Intestinal Phosphate (CIP) prevented the digested fragments from self ligating. For CIP treatment, the sample was spun down and 0.5μl vector DNA CIP (0.5unit/μg, provided by NEB) was added. After an incubation for 60' at 37°C the enzyme was heat-inactivated at 65°C for 20' and then the vector was gel-purified following the QIAquick PCR Purification Kit (Protocol using a microcentrifuge) and finally eluted in 30μl EB buffer. The concentration of the digested plasmid was measured by loading in parallel part of the sample and a 1kb DNA ladder on a 1% agarose gel. This ladder could be used for approximating the mass of DNA by comparing the intensity of bands of similar sizes, since the approximate mass of DNA in each of the bands in the ladder, assuming a 0.5 microg. loading, was known.

5.2.2.2 Creation of the V5-6His fragment

First, a PCR was performed with the circular pYes2.1/V5-His/LacZ vector as a template. The upper primer was designed to anneal to the V5 epitope introducing a BamH1 cutting site, while the lower primer had to include a Sal1 recognition site.

f-primer: GCGGATCCGGTAAGCCTATCCCTAACCCTC
BamH1 V5 epitope

r-primer: TGACGTCGACCTACACCGAACTGAGATAC
Sal1

8μl of the vector (~3pg/μl) was diluted in 30μl ddH2O together with 5μl Taq buffer (PROMEGA) and 1μl dNTP's. Then 2.5μl of the f- and r-primers were added, as well as 1μl of Taq polymerase (5U/μl) (PROMEGA). The PCR was performed as followed: 95°/1' <(95°/15", 60°/20", 70°/1'30'')x35> 70°/5'.

Afterwards the product was purified with the QIAquick PCR Purification Kit (Protocol using a microcentrifuge) and eluted in 30µl EB buffer. Following purification, the PCR product was cut with BamH1 and Sal1 and purified again using the same procedure as described for the digestion of the vector.

5.2.2.3 Generation of the bait vector

The digested plasmid, serving as vector, had to be ligated with the PCR product, being the insert. To increase the possibility that only one insert was cloned into one vector the DNA concentrations for the ligation were chosen 1:3 (Cvector/3797bp: Cinsert/816bp). Therefore, the reaction mix consisted of 2µl ligation buffer, 1µl T4 DNA Ligase (1U/ul) (MBI Fermentas), 2µl (65ng) insert DNA, 1µl (100ng) vector DNA and 14µl ddH2O. The mix was left at room temperature for 45', then put o/n at 16°C.

After *E.coli* transformation, control restriction and control PCR, the direct sequencing confirmed the in-frame fusion of the V56His fragment to the backbone. The amplification of the new bait vector followed the description given on 5.2.1 with competent cells kindly donated by Renjie Jiao.

5.2.3 Construction of the prey vectors

The aim was to replace the 6His flanking the tags of the prey (3HA, 2MYC, 3VSV) in the pU6H plasmid series with a V5 epitope due to two reasons: the 6His was already used as a tag for the bait vector and the 5-prime end of the prey's tag had to be identical to the one of the bait, thus minimizing the construction of primers needed for the generation of both yeast libraries. The strategy was identical to the one described for the bait vector apart from the following variations:

- For the digestion of the vectors (pU6H2MYC, pU63HA and pU6H3VSV) BamH1 and Pst1 were used as restriction endonucleases.

- The 5' primer had to anneal to the specific tag and contain a V5 epitope, as well as a BamH1 restriction site, while the 3' primer had to start after the already existing Pst1 region in the plasmid template.

f-primer (3HA): GCGGATCCGGTAAGCCTATCCCTAACCCTC
BamH1 V5 epitope
TCCTCGGTCTCGATTCTACGGGATACCCGTATGATGTTCCG

f-primer (2MYC): GCGGATCCGGTAAGCCTATCCCTAACCCTC
BamH1 V5 epitope
TCCTCGGTCTCGATTCTACGGGAGAGCAGA AATTGATCAGC

f-primer (3VSV): GCGGATCCGGTAAGCCTATCCCTAACCCTC
BamH1 V5 epitope
TCCTCGGTCTCGATTCTACGGGATACACCG ATATCGAGATG

The r-primer was binding to the Kan^r cassette generously given by Graziella Pedrazzi.

5.2.4 Creating the bait and prey DNA libraries

5.2.4.1 Construction of the primers

Three specific primers had to be created for each ORF: a forward and a reverse primer encompassing the tag, as well as the Kan^r, and a specific forward control primer.

Forward primer for endogenous tagging

The forward primer consisted of 40bp of homology to the ORF to be tagged. These nucleotides were located directly upstream of the ORF's stop codon. The homology to the ORF contained carboxy terminal amino acid sequence of the ORF excluding the stop codon. At the 3' of the forward primer 20bp were added, which annealed to the V5 epitope. This generated a length of 60 nucleotides.

Reverse primer for endogenous tagging

The 5' part of the reverse primer consisted of 40bp, which annealed to an area approximately 100 basepairs downstream of the stop codon and 20bp annealing downstream of the Kan^r cassette located within the plasmid.

Control primers

To check for proper integration of the cassette into the genome, an additional forward primer was designed being 20bp long, containing at least a total amount of 10 G+C (annealing temp: 60°C) and being located approximately 400bp upstream of the stop codon. Each forward control primer was ORF specific, whereas the reverse primer for the control PCR annealed downstream of the Kan^r and could, therefore, be used for all control PCR reactions.

A fragment of expected size (# of nucleotides starting from the 5' primer to the beginning of the stop codon + the constant bp product of the cassette [being 554bp for the His-, 452bp for the VSV-, 410 for the MYC- and 435 for the HA- tag]) would only be produced by this control PCR if the tagging cassette was inserted behind the targeted gene.

CKA1f	ACACCCGTGGTTTGCCCCAATAAGGGAA CAAATTGAAAAAAggtaagcctatccctaaccc
CKA1r	CCTCTGTTGTATGGAGGAAGCCCTTTAAA CCTGTATTTAGccccgcgcgttggccgattc
CKA1cf	GAACGAGTAACACAGACCAG
Cr	GAGCGTTTCCCTGCTCGCAG

Table 1. Primer set for CKA1.
ggtaagcctatccctaaccc annealing to the V5 epitope
ccccgcgcgttggccgattc annealing to the Kan^r cassette

5.2.4.2 Generation of the in vivo recombinants

PCR using a 50µl Rxn containing 38µl of ddH2O, 5µl of Pfu buffer (10x), 1µl of dNTP's (10mM), 1.25µl of the forward and reverse primer (20pmol/µl) and 1µl of the proper template (~50ng bait or prey) was performed. After adding 2.5µl of the polymerase (Taq [5U/µl]: Pfu [3U/µl]: H2O = 1:1:14), 35 PCR cycles were performed (95°/15", 60°/20", 70°/2) starting with an initial denaturation of 95°C for one minute and ending with the final elongation at 70°C for 5 minutes.

Each PCR product was tested by running 2µl of sample on a gel.

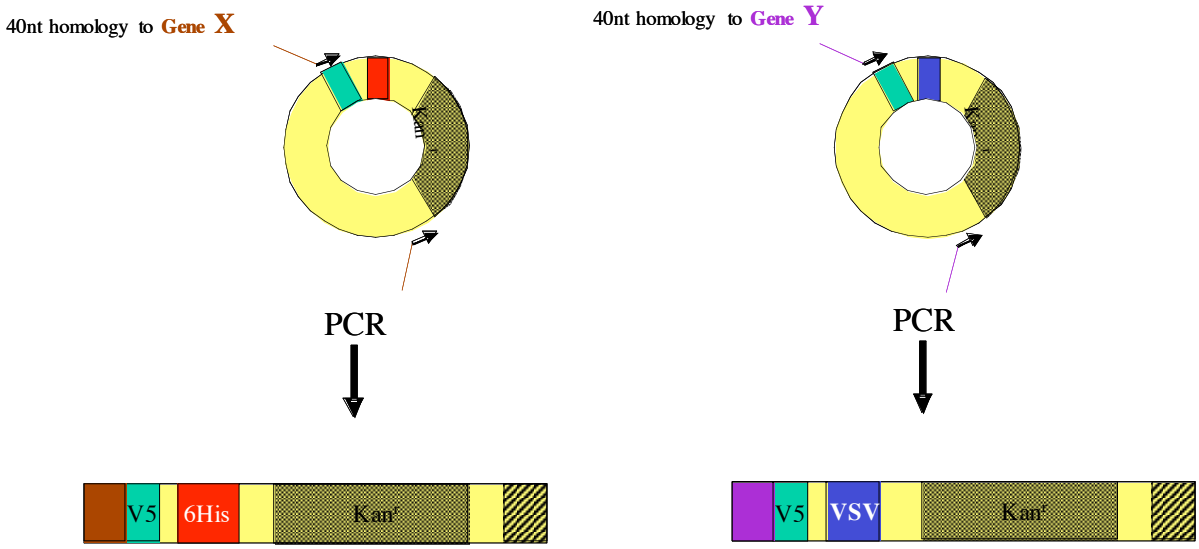

Figure 4. Construction of the bait (Gene X) and prey (Gene Y) PCR fragment. For both vectors, the last 20bp of the forward and reverse primers were similar, annealing to the V5 epitope (f-primer) and to the Kan^r cassette (r-primer). However, the first 40bp of the primers were gene specific, creating a PCR fragment encompassing the bait or prey tag, ready for insertion into the genome.

5.2.4.3 Yeast transformation

Once the PCR product containing the V5 epitope, the appropriate tag, Kan^r and the homologies to the ORF was produced, it could be transformed into the yeast cells. Upon transformation into the yeast, the areas of homology between the PCR product and the genome would align allowing recombination to occur and thus integrating the tag into the genome.

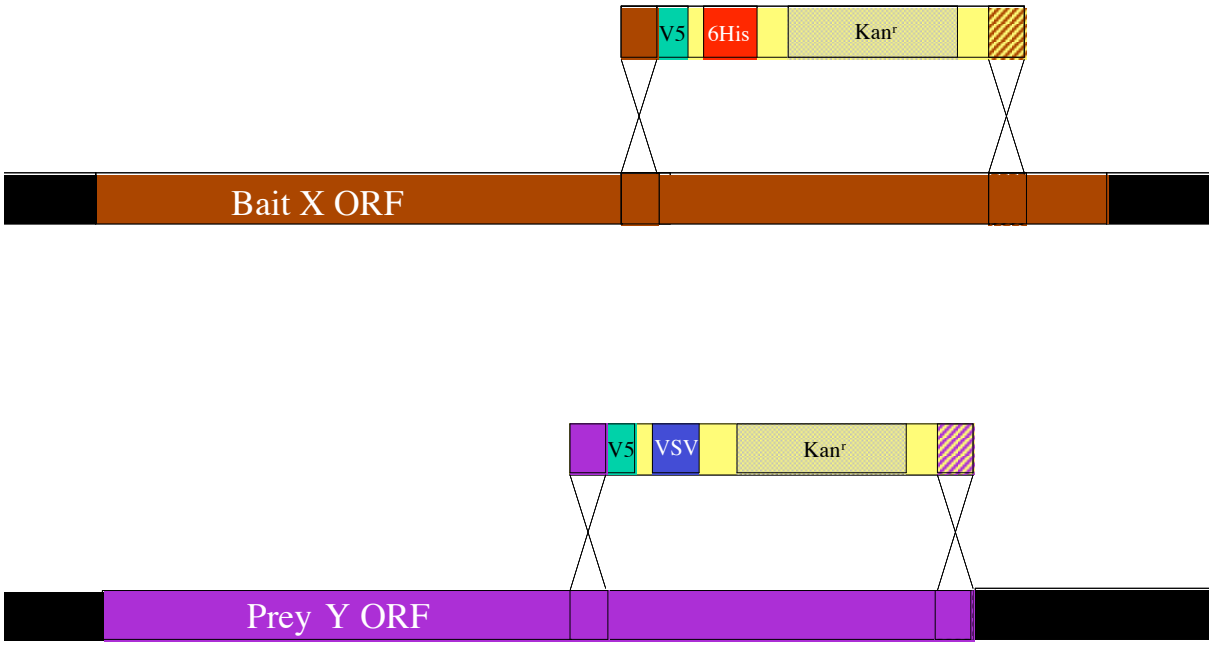

Figure 5. Yeast transformation of the PCR products. Since the PCR fragment was flanked at 5' and 3' by 40bp homologous to the ORF, it could get inserted into the genome of the yeast by homologous recombination.

The transformation of the cells was performed essentially by modifying the lithium acetate method (Agatep *et al*., 1998). An overnight culture of the proper yeast strain, a-strain for the bait; **BY4742** (MATα ura30 leu20 his31 lys20), α-strain for the prey; **BY4741** (MATa ura30 leu20 his31 met150), was incubated in 5ml YPD at 30°C in a shaker at 200rpm. The OD of the culture was measured and diluted to an OD of 0.1 in a final volume of 40ml, which provided enough cells for 8 different transformations. The new culture was incubated at 30°C, for about 3 hours, till a cell density of 2x107/ml, equivalent to an OD 600 of 0.4, was reached. Then the cells were harvested in a sterile centrifuge at 4°C by 3500rpm for 4 minutes. The medium was poured off, the cells resuspended in 25ml ddH2O, recentrifuged and diluted in 1.0ml 100mM LiAc as an additional washing step. After a spin down of 15 sec the pellet was suspended in 400μl of 100mM LiAc which contained about 2x109 cells/ml. After splitting into 50μl aliquots, giving the proper amount for one transformation, the experiment could either be stopped and the competent cells frozen and stored at –80°C (in a NALGENE Cryo 1°C Freezing Container; rate of cooling –1°C/minute) or proceeded with right away.

The transformation mix consisted of 45μl PCR product, 240μl PEG (50%w/v), 6μl 1.0M LiAc, 10μl ssDNA (10mg/ml) and 29μl ddH2O. All of the ingredients were added on ice in the ordered list so that the PEG could shield the cells from the detrimental effects of the high concentration of LiAc, then vortexed vigorously until the cell pellet had been completely mixed, which took approximately 1 min. After 30 min incubation at 30°C and 30 min heat shocking at 42°C, the cells were collected in a microcentrifuge at 8000rpm for 15 sec and the supernatant was sucked off with a micropipette. Now the cells were resuspended in 1ml of YPD and transferred into a 15ml falcon tube containing 4ml of YPD. After an incubation at 30°C by 250rpm for 2.5 hours the mixture was spun down again for 3’ at 3500rpm to remove the supernatant. To resuspend the pellet, 800μl of ddH2O was added. Then the cells were plated out onto 2 big Geneticin G418 plates (145/200, Greiner), which served as a positive selection, allowing only those cells to grow which had integrated the Kan^r into their genome. The incubation of the plates at 30°C lasted 3-4 days, till the growing colonies had reached an appropriate size.

The last step consisted of streaking out the promising colonies onto new G418 plates and letting them grow again for one day in the incubator to ensure that only one colony was growing on each plate.

5.2.4.4 PCR colony screen

This additional control confirmed that the cassette had integrated into the proper place in the genome. To accomplish this, a PCR reaction was performed using an ORF specific control forward primer (strategy described in 5.2.4.1 Construction of the primers) and a constant control primer towards the Kan^r. The only way that this particular PCR reaction could produce a fragment of the expected size was if the PCR tagging cassette had inserted behind the gene to be tagged.

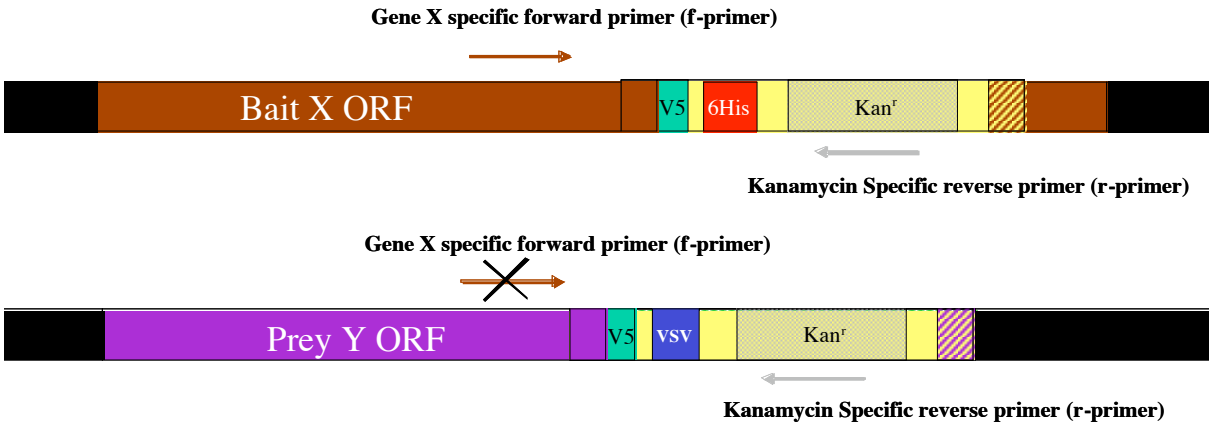

Figure 6. Colony PCR to confirm the proper insertion of the tagging cassette. Since the f-primer is gene specific and only the r-primer anneals to the inserted cassette, a PCR product of the expected size could only be obtained if the cassette was integrated into the selected ORF X.

The mastermix was prepared as followed: to 5μl Mg2+ free Taq Buffer (Promega), 3μl Mg2+ (25mM), 0.7μl reverse constant primer (20pmol/μl), 0.5μl dNTP's (10mM) as well as 41.6μl ddH2O was added. Then 49.3μl of the mastermix was pipetted into a 1.5μl eppendorf tube containing 0.7μl of the corresponding ORF specific forward primer. A small amount of the colony to be screened was picked using a 200μl plastic tip pipette, mixed well into the PCR mixture and incubated at 100°C for 5' in order to crack the cells open. To bring down the condensation afterwards, the tube was quickly spun before being cooled on ice for 5-10 minutes. At the end, the lysate was transferred into a PCR tube and 0.75μl (5U/μl) of Taq polymerase (PROMEGA) was added. The PCR was made at 95°/1' <(95°/15", 52°/20", 70°/2')x35> 70°/5' and 5μl of the PCR product was run on a gel to verify its length.

When the result was positive a small amount of the colony was picked and stored at –80°C in 1ml of YPD containing 15% of Glycerol as a stock.

5.2.5 Set up of LiquiChip technology

5.2.5.1 Mating of a specific bait and prey strain

In order to study a supposed protein interaction, the two genes of interest first had to be brought into one cell. Since the bait strains were a-type haploid cells and the prey strains α-type haploids, the bait and prey lines could be mated and selected for diploid a×α cells which expressed both: bait and prey tagged proteins.

One loop full of the freshly grown (see 5.2.4.3 Yeast transformation) desired a- and α-strain were inoculated into 5ml of YPD medium and put o/n at 30°C with 250rpm shaking. The particular α-type strain used was able to produce the aminoacid Lysine (*Lys*$^+$) but could not produce the aminoacid Methionine (*Met*$^-$) which it had to obtain from the medium in order to survive. The a-type (*Met*$^+$, *Lys*$^-$) strain required the opposite aminoacid situation for growth. Therefore, when grown on medium which lacked both Lys and Met, only the diploids were able to synthesize these two aminoacids and, therefore, survive.

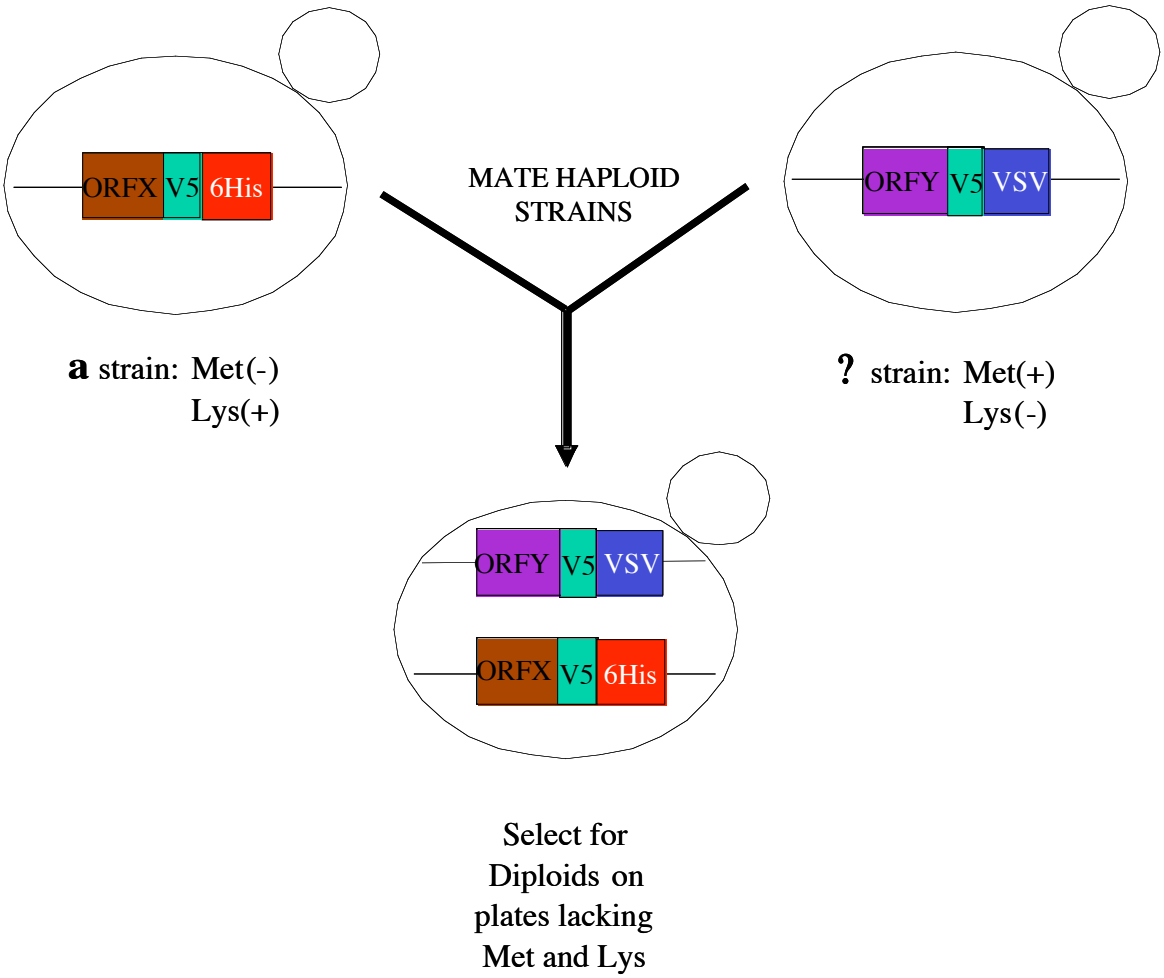

Figure 7. Mating of the a- and α-strain. The two haploid yeast strains containing each a specific gene, bait X (a-strain) or prey Y (α-strain), are mated to get diploid yeast cell lines expressing both tagged proteins. The selection on Met^- Lys^- media assures that only those diploids, having both tagged ORFs in their genome, are able to grow.

Thus 100µl of ddH2O with 5µl of o/n culture was streaked out on a -Met/-Lys plate to select for diploids. From the diploid plate, 5ml of -Met/-Lys media was inoculated with diploids and grown o/n at 30°C with 250rpm shaking. The OD was measured at 600λ, and diluted to an OD of 0.3 in a final volume of 25ml of fresh -Met/-Lys solution and incubated for about 3 hours till an OD600=1 was reached. Then the fraction was spun down for 5' at 3000rpm to get rid of the supernatant and the cell pellet was either immediately frozen and stored at –80°C or directly proceeded with for protein extraction.

5.2.5.2 Protein extractions

Since yeast cells have a complex proteinaceous cell wall, which makes lysis difficult, two types of extraction methods were tested for their feasibility and compatibility with the LiquiChip system.

The first method used the mechanical action of small glass beads to shear the cell wall. In order to do so, 6ml of Breaking buffer was prepared by adding 6µl of protease inhibitors B, P, L (1µg/mL) and 120µl PMSF (200mM). 5ml of Breaking buffer and protease inhibitors were added to the cell pellet from 100ml of yeast liquid culture and centrifuged at 4°C with 3000rpm for 5'. After discarding the supernatant, the remaining 1ml of buffer was added to resuspend the cells. The lysate was transferred into 2 eppendorf tubes, each containing 200µl of glass beads. The tubes were vortexed for 20" and then cooled down on ice for 1'. This step was repeated 8 times before centrifuging at 13,000rpm for 15' at 4°C in a benchtop centrifuge. The supernatant was transferred into a new tube and spun down again for 20' to get rid of the remaining beads. Then, the supernatant was pipetted into another eppendorf, and 5µl was taken to measure the protein concentration, while the rest was immediately frozen in liquid nitrogen and then stored at -80°.

The second method for cell lysis relied on a commercially available chemical, Y-PER (Pierce), to break the cell wall and thus release the soluble proteins. To do so, 2.5ml of Y-PER containing 2.5µl B, P, L and 100µl PMSF (100mM) was added per 0.5g of yeast pellet and vortexed thoroughly. The tube was then agitated on a roller at room temperature for 40-60 minutes and then centrifuged at 4°C for 10' at 3000rpm. The liquid was transferred into another tube, 5µl were used to measure the protein concentration, and the rest was frozen in liquid nitrogen and stored at -80°.

The protein concentration was assayed by the method of Bradford (Bradford, 1976), buffer and protein standards provided by BIO-RAD.

5.2.5.3 Western blot analysis

To specifically show the expression of the tagged proteins western blotting was performed for those protein extracts, which were later used for experiments with the LiquiChip. Therefore, the proteins were separated by SDS-PAGE (Laemmli, 1970) and subsequently transferred to a nitrocellulose membrane (OSMONICS) using a wet transfer for 1.5 hours at 100V. To saturate the remaining binding sites on the nitrocellulose, the membrane was blocked in TBS containing 5% milk for 1-1.5 hours, left on a rolling wheel at room temperature. After this, the membrane was incubated for 1-2 hours in TBST containing 2% milk as well as the primary antibody. For detection of both, the bait and the prey proteins, a mouse α-V5 (1:5000) was used. In order to detect the bait and the prey separately, a PentaHis antibody (1:1000) and a mouse α-VSV (1:400) were used respectively. After washing 2 times with TBST for 5', the membrane was exposed for 1 hour to the secondary antibody, anti-mouse IgG (1:5000), diluted in TBST. Further washing was performed 3 times with TBST and the last time with TBS for a duration of 5' for each step. The bound antibodies could be detected using the ECL system (Enhanced Chemiluminescence, PIERCE) and visualised by exposing the membrane to an X-ray film. If the result was not satisfying, due to the low limit of detection of the ECL, detection was redone with the more sensitive FECL (PIERCE).

5.2.5.4 Pull downs

To test the binding of the bait to the beads as well as to the prey, pull downs were performed with the extracts for use with the LiquiChip. First, 100μL of the Ni-beads (Probond^{TM}, Invitrogen) were washed for 5' with 2x3ml of Incubation buffer on a roller at 4°C followed by a spin down at 4000rpm for 2 minutes at the same temperature. Then, the glass bead produced protein extracts (from a starting culture of 40-50ml) were added to the Ni-beads. After an incubation of 2 hours at 4°C on a roller, the Ni-beads were washed with 3ml of Incubation buffer for 5' on a roller followed by a spin at 4000rpm for 2' to bring down the beads. The washing step was performed 4 times at 4°C. Afterwards, the sample was eluted with 50μl of Elution buffer and 5μl of Loading buffer before being boiled at 100 °C for 5'. Then, the tube was spun down at 4000rpm for 2' at 4°C in order to remove the supernatant before freezing it at -20°C or proceeding right away by running a gel and making a transfer.

After the transfer, Ponceau was added and the lanes were marked with a pen and the blot documented before the blocking was performed in 10% milk/TBST for 1 hour at room temperature. Next, an over night incubation in the cold room took place with the primary antibody (5%milk, 1:5000 mouse anti-V5). The next day, the membrane was washed for 2 hours with TBST, which got changed at least four times, before the incubation with the secondary antibody (2%milk) was made for 1 hour at room temperature.

5.2.5.5 LiquiChip machine

Several parameters allow protein-protein interactions to be detected by the LiquiChip machine. The machine uses two lasers, a red laser (645nm), which detects the fluorescently labeled beads (bound to the bait tag) and a green laser (532nm), which is able to detect the fluorescently labeled antibody (bound to the prey tag).

Once samples were loaded into the LiquiChip machine, a stream of sample fluid carried the beads to a very narrow tube allowing only a single file passage of beads. The alignment of the beads within the passage enabled single beads to be irradiated by both lasers at the point where the red and green lasers intersected. At this point, fluorescent signals generated from the beads and the reporter molecules were simultaneously recorded. In this set-up, 100 individual beads could be analyzed for the presence or absence of the fluorescently labeled antibody. From this averaged data, the protein interaction status of the bait and prey could be indirectly determined. Furthermore, since the fluorescent antibody signal was only recorded in conjunction with the bead fluorescence, signals from free, unbound fluorescently labeled antibodies were not recorded.

The machine was programmed to suck up 100 microliters of each sample and then count 100 beads (with the red laser) within 100 seconds or the test failed. The output the machine gave is called MFI (Mean Fluorescence Intensity), the measurement of the green fluorescence. In order to know the validity of the MFI a negative control was present at all times.

5.2.5.6 Assays

The basic assay consisted of 200µl BSA/PBS buffer containing 0.2µl B, P, L (1µg/µL), as well as 4µl PMSF (200mM), 1µl beads (2500 beads) and approximately 25µg of the protein extract to be tested, incubated on a shaker at 4°C in the dark o/n. Then 50ng of the primary antibody was added and an incubation of 2 hours took place before adding 200ng of the secondary antibody. After another incubation of half an hour at 4°C on a roller in the dark, the samples were run in the LiquiChip machine.

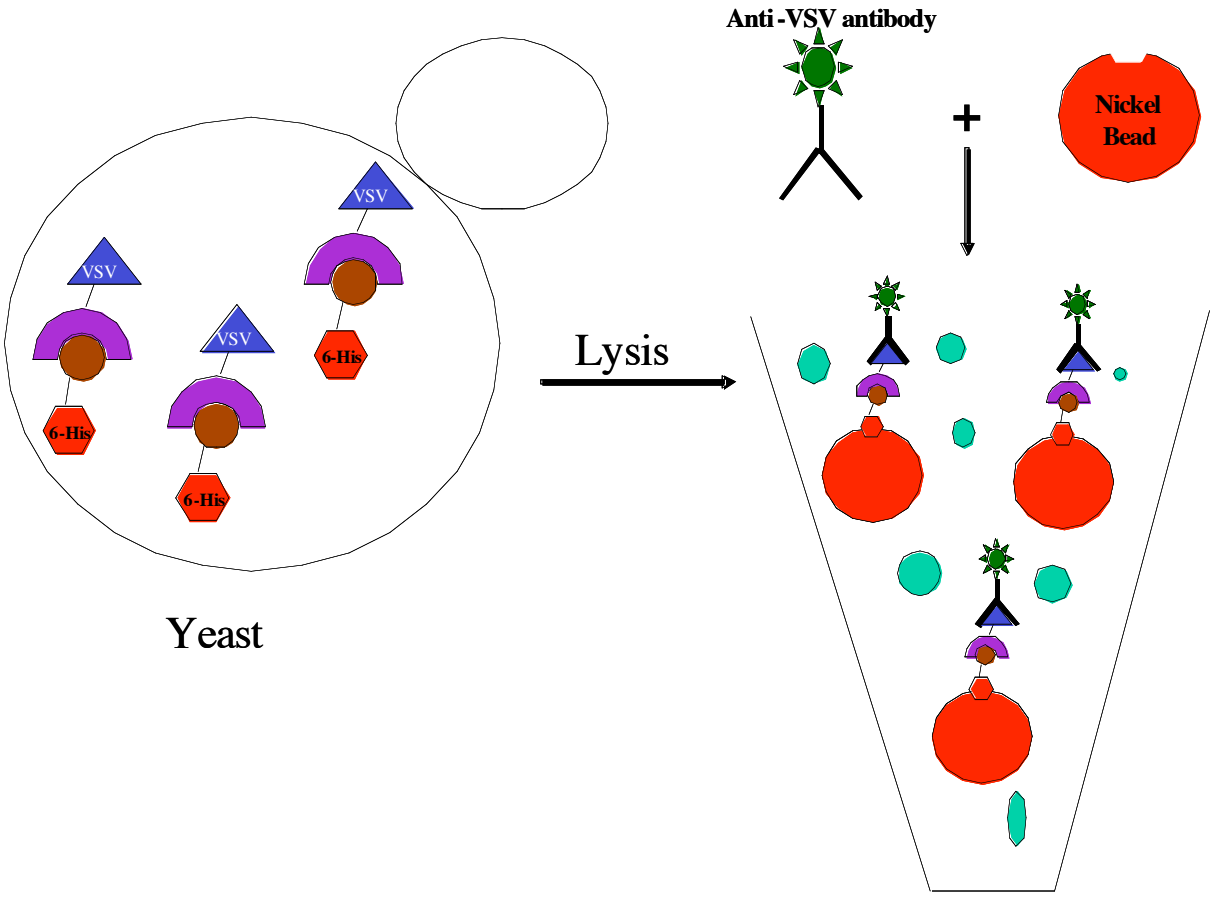

Figure 8. LiquiChip assay. The protein extracts contained the bait and the prey tagged proteins. While the beads annealed to the 6His tag, the antibodies bound to the tag of the prey.

6 Results

6.1 Arrays

One goal of this project was to create a system to endogenously tag all proteins involved in DNA replication, repair and recombination with two different epitope tags in a simple, rapid and efficient manner.

After attempting to tag 488 ORFs as both bait and prey, giving a total number of 976, a final array of 927 endogenously tagged ORFs was accomplished consisting of 470 baits and 457 preys.

6.1.1 Creation of the vectors

Two different vectors were needed to generate the assay: a bait and a prey vector were used as a template for PCR to produce "PCR tagging products", which were transformed in a- and α-cells respectively. Important was that the bait and prey vectors only differed in the tag itself. Therefore, primers required for producing the "PCR tagging product" were interchangeable on bait and prey vectors, thus reducing the number of primers needed for the tagging of each ORF. By positioning a V5 epitope 5' to the specific bait and prey tag, a common f-primer site was given for both plasmids. The Kan^r at the end of the cassette served as a positive selection marker for the strains having integrated the tag, when grown on a YPD plate containing G418.

For the bait, the V5-6His fragment of the pYes2.1/V5-His/LacZ was cloned into pU6HMYC resulting in the pUV5-6His vector. The introduction of the V5-3HA, V5-2MYC and V5-3VSV PCR product into the pU6H-3HA, pU6H-2MYC and pU6H-3VSV created the three possible prey vectors, as to be pUV5-3HA, pUV5-2MYC and pUV5-3VSV (see Materials and Methods).

After endogenously tagging 15 ORFs, the pUV5-3VSV vector was elected to be the prey vector of choice since the VSV tag produced the best signal for western blotting (data not shown).

6.1.2 Bait and prey library

Endogenously tagged ORFs were produced in α- and a-format for 488 genes. Several control steps were undertaken to reassure the reliability of the produced libraries:

G418 resistancy

The transformed strains were grown on G418 plates allowing only the cells with a Kan^r gene to grow as a confirmation of the integration of the cassette into the genome.

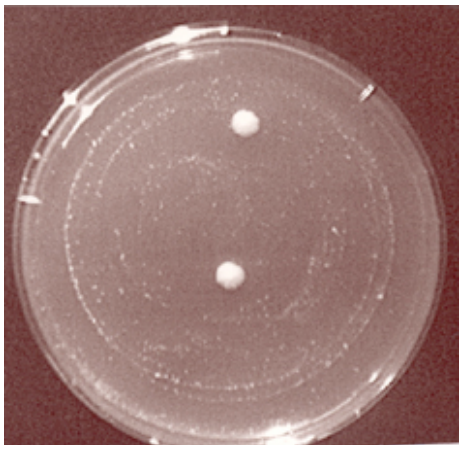

Figure 9. G418 resistancy. Kan^r colonies growing on a G418 plate as a control for the insertion of the tag into the genome.

Colony screen

The correct insertion of the tag into the genome was verified for each strain by performing a colony screen and verifying the expected size of the PCR product. The expected colony PCR product was equal to the number of nucleotides starting from the 5' control primer to the beginning of the stop codon, adding the constant bp product of the cassette (being 554bp for the His- and 452 for the VSV- tag).

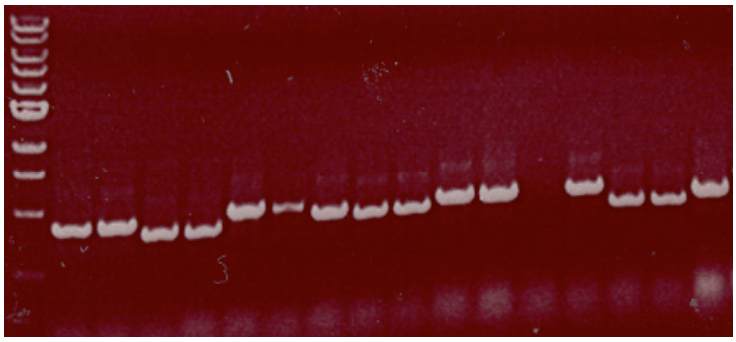

Figure 10. Colony PCR products. 5µl of the PCR product was loaded per lane on an agarose gel. The size of the PCR product could be estimated by comparing its band size to the 1kb DNA ladder.

Western blot

In addition, all ORFs later worked with on the LiquiChip were tested for their correct expression by performing a western blot. There were two different options concerning the primary antibody: either an anti-V5 antibody could be used for both, the baits and the preys, or a distinct antibody could serve for the detection of the tags, being PentaHis antibody for the baits and anti-VSV for the preys. After an incubation with anti-mouse IgG as a secondary antibody, the bound antibodies got detected by ECL. CKB2, with an expected size of 34kD, served as a positive control for all testings.

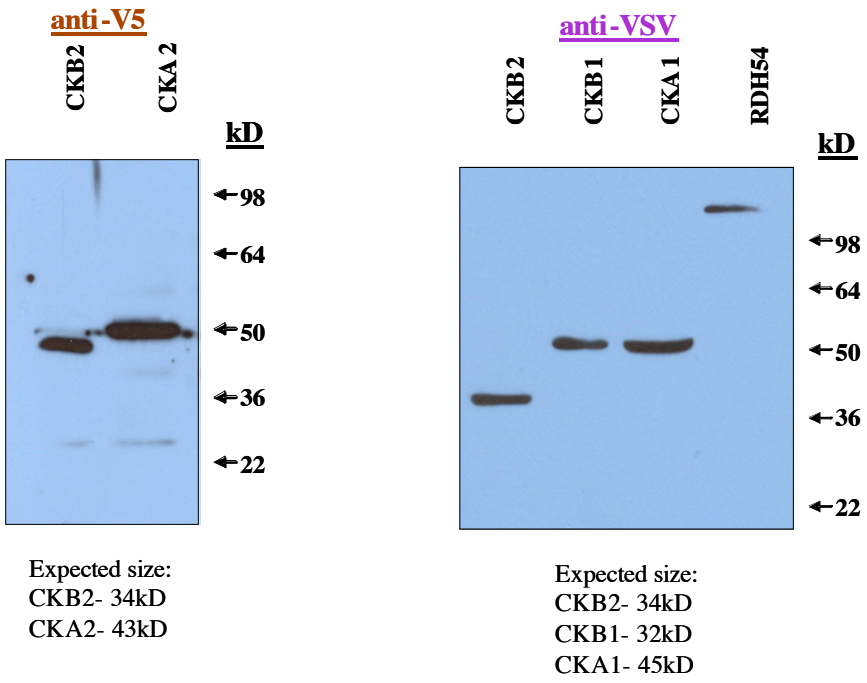

Figure 11. Western blot of baits and preys. The anti-V5 antibody was taken as a primary antibody for the detection of the bait CKA2. The preys CKB1, CKA1 and RDH54 were detected by an anti-VSV antibody. In both cases CKB2 served as a positive control, either with a His tag for the baits or with a VSV tag for the preys.

Sequencing

All ORFs were sequenced to confirm the correct tagging on the genetic level. Of a total number of 1048 colony PCR products 80% of the PCR sequence was as expected while 20% either contained mutations in the ORF, V5, 6His or 3VSV domain, or the sequencing run did not work.

6.2 LiquiChip assay

Several steps had to be undertaken before protein interactions could be tested by the LiquiChip technology. One part consisted of verifying the compatibility of the created libraries with the beads used in the LiquiChip, the other was getting familiar with the handling of the machine, and the last was adapting and optimizing the assays using yeast crude extracts. To insure the reliability of the results, distinct protein pairs had to be chosen serving as positive and negative controls and each test was carried out at least two times.

For the positive controls CKA2 was taken for the baits and CKA1 for the preys. These proteins are part of the Protein Kinase II, also called Casein Kinase II, which consists of a tetramer of two α- (CKA1 and CKA2) and two β- (CKB1 and CKB2) subunits (Glover *et al.*, 1998). The interaction has been confirmed by three different biochemical methods: affinity precipitation (Krogan *et al.*, 2004; Ho *et al.*, 2002), two hybrid (Uetz *et al.*, 2000) and the analysis of purified complexes (Cardenas *et al.*, 1993).

Baits (His) \ Preys (VSV)	CKA1	CKA2	CKB1	CKB2
CKA2	Affinity purified - Ho *et al.*, 2002; Gavin *et al.*, 2002 Two Hybrid - Uetz *et al.*, 2000 Purified complex - Cardenas *et al.*, 1993		Synthetic Lethal - Bidwai *et al.*, 1993 Two Hybrid - Uetz *et al.*, 2000 Purified complex - Cardenas *et al.*, 1993 Affinity purified - Ho *et al.*, 2002; Gavin *et al.*, 2002	Affinity purified - Ho *et al.*, 2002; Gavin *et al.*, 2002 Synthetic Lethal - Bidwai *et al.*, 1993 Two Hybrid - Uetz *et al.*, 2000; Ito *et al.*, 2001 Purified complex - Cardenas *et al.*, 1993
CKB2	Affinity purified - Ho *et al.*, 2002; Gavin *et al.*, 2002 Two Hybrid - Uetz *et al.*, 2000 Purified complex - Cardenas *et al.*, 1993	Affinity purified - Gavin *et al.*, 2002 Synthetic Lethal - Bidwai *et al.*, 1993 Two Hybrid - Uetz *et al.*, 2000; Ito *et al.*, 2001 Purified complex - Cardenas *et al.*, 1993	Affinity purified - Ho *et al.*, 2002; Gavin *et al.*, 2002 Two Hybrid - Uetz *et al.*, 2000 Purified complex - Cardenas *et al.*, 1993	

Table 2. Protein Kinase II family. By tagging all the members of the Protein Kinase II family, a variety of well documented protein interaction pairs were available giving a solid platform for the testing of the LiquiChip machine.

As a negative control REM50, a regulator of theTy1 transposition, was chosen since it is involved in the regulation of a mitochondrial network (Huh *et al*., 2003) and, therefore, it is unlikely to be an interacting partner of the positive controls or of any of the proteins involved in DNA repair, replication and recombination.

6.2.1 Pull downs

Several conventional pull downs were made to prove the binding of the His tagged proteins to the nickelbeads as well as to the VSV tagged proteins.

The CKA2 (bait) × CKA1 (prey) diploid served as a positive control. As a negative control, diploids containing only the CKA1 (prey) were tested to eliminate the possibility of the CKA1 (prey) nonspecifically binding to the nickelbeads.

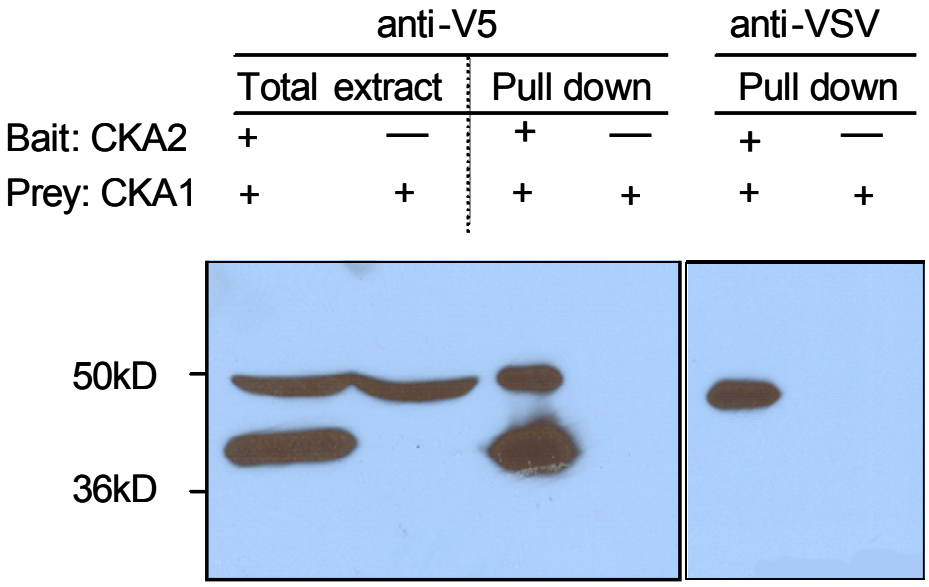

Figure 12. Pull down between CKA2 (bait) and CKA1 (prey). The CKA1 (prey) only associated with the beads in the presence of the CKA2 (bait). This suggests that CKA1 (prey) association with the beads is CKA2 (bait) dependent and that the proteins interact. Therefore, the CKA2×CKA1 pair could serve well as a positive control.

6.2.2 Fen1/ Thioredoxin

To get familiar with the handling of the LiquiChip system, the first trials were made with Thioredoxin, a purified, biotinylated 6His-tagged non-yeast protein, as well as Fen1, which was not biotinylated and, therefore, needed a fluorescent antibody in order to be detected by the LiquiChip machine. The aim was to figure out the best buffer conditions, the optimal amount of antibodies needed, the most appropriate beads as well as the fluorescent signal level (MIF), which best represented a positive result.

6.2.2.1 Binding of Fen1 to the Ni-Beads

To test the binding of the 6His Fen1 protein to the beads, purified Fen1 was incubated with the beads and then detected by a primary rabbit α-Fen1 antibody followed by a fluorescent α-rabbit secondary antibody. As negative controls, samples without Fen1, or the Fen1 rabbit antibody, were tested. These samples where then analyzed by the LiquiChip machine. A high MIF indicated an association between the red fluorescence of the Ni-Beads and the green fluorescence of the secondary antibody.

1AB: rabbit α-Fen1

2AB: α-rabbit (fluorescenced with Alexa532)

SAMPLE	BUFFER	PROTEIN	NI-BEADS	1 AB	2 AB	RESULT
S1 (pos)	200 microl	5 ng	2 microl	50 microl	200 microl	1026.50 MIF
S2 (neg)	200 microl		2 microl	50 microl	200 microl	246.50 MIF
S3 (neg)	200 microl	5 ng	2 microl		200 microl	20.06 MIF

Table 3. Binding of Fen1 to the Ni-Beads.

There was a big difference in the MIF between the positive control (S1) and the two negative controls (S2 and S3). The high MIF of S1 showed that there was a binding of the Fen1 to the Ni-Beads, an annealing of the 1AB to Fen1 as well as a binding of the 2AB towards the 1AB. Since there was no protein in S2 the beads should not bind to the antibodies, and, therefore, no fluorescencing signal should be produced. However, the MIF of S2 suggested that some unspecific binding of the antibodies to the beads occurred. S3 demonstrated that the signal decreased dramatically if no primary antibody was added. These two MIFs we judged as background.

6.2.2.2 Binding of Fen1 to different beads / Influence of empty yeast lysate

This test was performed to find out which of the two beads that came into consideration suited better to our assays. The advantage of the Ni-NTA-Beads was their high binding capacity (2ng of protein per 0.5 microl of beads). However, they were not very specific in binding to the His-tag. In comparison, the Penta-Beads were very specific in their binding to the His-tag due to their layer of Penta-His-Antibody (mouse monoclonal IgG1), while their binding capacity was low (1ng of protein per 0.5 microl of beads). By testing the two beads in one set-up, the same conditions were assured, and, therefore, the MIF could be taken as a reference to choose the beads to proceed with.

In addition, two more samples, containing empty yeast lysate, were added to see what influence the yeast lysate "background" had on the MIF. A significant drop of the MIF would indicate that the lysate either interfered with the binding of the Fen1 to the beads, or to the antibodies or that it would interfere with the detection of the Alexa534 signal.

Four samples were made, two containing only beads and the other two contained yeast lysate as well. Fen1 was incubated o/n in the mix and then detected by the primary rabbit α-Fen1 antibody followed by the fluorescent α-rabbit secondary antibody.

1AB: rabbit α-Fen1

2AB: α-rabbit (fluorescenced with Alexa532)

Ni-Beads: Ni-NTA-Beads

P-Beads: Penta-Beads

Lysate: empty a-lysate

SAMPLE	PROTEIN	NI-BEADS	P-BEADS	LYSATE	RESULT
S1 (pos)	5 ng	3 microl			2639.00 MIF
S2 (pos)	5 ng		3 microl		282.00 MIF
S3	5 ng	3 microl		100 microg	61.00 MIF
S4	5 ng		3 microl	100 microg	45.00 MIF

Table 4. Binding of Fen1 to different beads.

S1 and S2 showed that the Ni-Beads had a higher binding affinity to Fen1 than the P-Beads since its MIF was almost 10x higher. S3 and S4 demonstrated that the lysate of the yeast decreased the MIF dramatically. It was not clear yet if this was due to unspecific binding of the antibodies to the lysate, if the lysate was disturbing the interaction of the protein and the beads or if the presence of the yeast was absorbing the fluorescent signal of the secondary antibody and, therefore, could not be detected as strongly by the green laser.

The test made clear that we had to proceed with the Ni-Beads because of its significantly higher MIF. Furthermore, the tests with the empty yeast lysate (S3-S4) indicated that we had to figure out where the lysate interfered in the pathway of the signaling cascade.

6.2.2.3 Pathway of disturbance of empty yeast lysate

To pinpoint the disturbance of the empty lysate we first had to reduce the number of interference possibilities to a minimum. For this reason we changed our testing protein to Thioredoxin. Its advantage consisted in the need of only one detection protein, called Streptavidin, which was already coupled to a fluorescent signal. From this, the possibility of a disruption between the binding of the two antibodies was already ruled out. The idea was to add the lysate at different steps of the assay to see if the incubation time was playing a role in the interference. Thioredoxin was incubated with the beads o/n, then Streptavidin was added and two hours later the samples were run on the LiquiChip. The empty lysate was either added to the sample from the beginning, at the same time as Streptavidin or just before the samples were put into the LiquiChip. Additionally, we wanted to confirm the results of the previous test considering the difference of the MIF caused by the two different beads, to make clear that the Ni-Beads were the beads of choice irrespective of the protein being used.

1AB: Streptavidin
Protein: Thioredoxin
Lysate: empty a-lysate (100microg)
o/n (over night)
w/1AB (together with 1AB)
b/m (before measuring)

SAMPLE	PROTEIN	NI-BEADS	P-BEADS	LYSATE	RESULT
S1	5 ng	1 microl		o/n	69.00 MIF
S2	5 ng	1 microl		w/1AB	483.00 MIF
S3	5 ng	1 microl		b/m	4922.25 MIF
S4 (pos)	5 ng	1 microl			6002.00 MIF
S5 (neg)		1 microl			19.25 MIF
S6	5 ng		1 microl	o/n	215.00 MIF
S7	5 ng		1 microl	b/m	1570.00 MIF
S8 (pos)	5 ng		1 microl		1411.00 MIF
S9 (neg)			1 microl		14.00 MIF

Table 5. Influence of empty yeast lysate.

The test was performed with Ni-Beads (S1-S5) and P-Beads (S6-S9). The signals were generally higher again when using the Ni-Beads confirming the result of the previous test. The MIF showed that the disturbance of the lysate was strongly connected with the incubation period. Its influence was basically negligible when added just before measuring. This made clear that its disturbance was not a matter of absorbing the fluorescenct signal but rather of interacting with the "set-up partners" of the protein. Since the MIF was extremely low when incubating the lysate o/n, the yeast extract definitely interacted with the beads or the Streptavidin in an unspecific way. Although the MIF increased when the lysate was added together with the Streptavidin, the signal was again almost 10x lower compared to when the yeast extract was added just before measuring.

6.2.2.4 Influence of empty yeast lysate according to amount

The fact that the tagged yeast proteins to be tested later on were non purified made clear that we had to inoculate the yeast lysate over night in all of the future assays. Therefore, the possibility to lower the negative influence of the yeast lysate on the MIF by changing incubation time was ruled out. So the only parameter we could possibly change was the amount of yeast lysate to be added. We hoped that by lowering its concentration we would be able to reduce the interference of the yeast lysate to a minimum. Therefore, we added different concentration of empty yeast lysate to Thioredoxin over night and then proceeded with the test as in the former set-up.

1AB: Streptavidin
Protein: Thioredoxin
Lysate: empty a-lysate

SAMPLE	PROTEIN	P-BEADS	LYSATE	RESULT
S1	5 ng	1 microl	100 microg	47.50 MIF
S2	5 ng	1 microl	50 microg	138.00 MIF
S3	5 ng	1 microl	25 microg	307.00 MIF
S4 (pos)		1 microl		3039.00 MIF

Table 6. Different amount of empty yeast lysate.

Sample 4, containing no lysate, served as a positive control. The first sample (S1) contained 100 microg of lysate, as in the test before. Then the lysate was lessened by one half in samples S2 and S3. The test made clear that the influence of the yeast lysate on the MIF strongly depended on the added amount. By lowering the amount of lysate, the MIF increase more than two folds.

6.2.3 CKA2

CKA2 (bait) was the first yeast protein to be chosen to work with on the LiquiChip since it should serve well as a positive control. However, the main problem was that the amount of CKA2 in the yeast lysate was not known, only the concentration of total protein extracted (Bradford method). Therefore, the first set-up had to consist of a competitive assay using other proteins of known concentration to be able to estimate the amount of CKA2 in the sample.

6.2.3.1 Binding of CKA2 to the beads in a competition assay

For this experiment, Thioredoxin (5ng) had to compete against two different His-tagged proteins of known concentration, Fen1 and PCNA-His. Their amount added to Thioredoxin was increased for three different concentrations to test if a competition for binding to the beads occurred between the proteins.

On the same time, empty lysate as well as CKA2 lysate was added. We expected to see a decrease in the MIF of the lysate containing CKA2, showing that this His-tagged yeast protein also competed with the Thioredoxin for binding to the beads. By comparing the MIF of CKA2 to the results of FEN1 and PCNA-His the binding strength of the yeast protein could be estimated and, like this, the total amount of lysate needed to reach a similar protein concentration.

To the purified proteins (S3-S8) the same amount of empty lysate (only 25 microg compared to 100 microg for the above experiments) was added as was for the CKA2 test, thus making the conditions as equal as possible. Since the MIF is a measure for the fluorescent signal surrounding each bead, reached by counting 100 beads on the LiquiChip machine, the MIF could only be high if Streptavidin, producing the fluorescence, was attached to the protein binding to the bead. Since Streptavidin

specifically recognized the biotinylated Thioredoxin, each binding of another protein to a bead would have lowered the signal and, therefore, the MIF.

1AB: Streptavidin
Proteins: S1-S9: Thioredoxin (5ng)
S3-S5: FEN1
S6-S8: PCNA-His
S9: CKA2 (a-lysate)
Ni-Beads: 1microl
Lysate: empty a-lysate (25 microg)

SAMPLE	LYSATE	FEN1	PCNA-HIS	CKA2	RESULT
S1 (pos)					839.00 MIF
S2 (pos)	25 microg				504.00 MIF
S3	25 microg	5 ng			216.00 MIF
S4	25 microg	10 ng			149.00 MIF
S5	25 microg	25 ng			110.00 MIF
S6	25 microg		5 ng		31.50 MIF
S7	25 microg		10 ng		23.00 MIF
S8	25 microg		25 ng		13.50 MIF
S9				25 microg	71.00 MIF

Table 7. Competition assay of Thioredoxin with FEN1, PCNA-His and CKA2.

S1 and S2 showed again the influence of the empty lysate on the measurement. Since S1 was only containing Thioredoxin the MIF was a lot higher than in S2, where the empty lysate interfered. For FEN1 and PCNA-His (S3-S8) the MIF steadily decreased when increasing their concentration. This proved that the proteins were binding to the beads competitively and that the high signal of the positive controls was due to specific binding of the beads as well as of the Streptavidin.

When testing 25 microg lysate containing CKA2 (S9) the MIF dropped dramatically showing that there was also a specific binding of CKA2 to the beads. The significant decrease of the MIF suggested that either the affinity of CKA2 towards the beads was a lot higher than the one of Thioredoxin or that the concentration of CKA2 in the lysate was extremely high.

6.2.3.2 Different concentrations of CKA2

With this test we wanted to find the best amount of CKA2 lysate for performing LiquiChip assays. For this reason we doubled the extract amount twice, hoping to see a change in the MIF. We started at our reference level of 25 micrograms in order to have a solid base of comparison.

An additional set-up was performed containing 5ng of Thioredoxin. This was basically the follow up of the previous competition assay. Since the MIF was produced by a secondary α-rabbit fluorescenct antibody connected to CKA2 by the primary rabbit α-V5 antibody, the signal this time was a measure for the amount of CKA2 bound to the beads. For validation of the data gathered, a negative control was included containing only Thioredoxin and empty lysate. Here, the MIF was expected to be extremely low since it only consisted of fluorescent background.

1AB:	rabbit α-V5
2AB:	α-rabbit (fluorescenced with Alexa532)
Proteins:	CKA2 (a-lysate)
	Thioredoxin
Lysate:	empty a-lysate
Ni-Beads:	1microl

SAMPLE	CKA2	THIORED.	LYSATE	RESULT
S1	25 microg			35.80 MIF
S2	50 microg			36.30 MIF
S3	100 microg			30.00 MIF
S4	25 microg	5 ng		15.30 MIF
S5	50 microg	5 ng		18.50 MIF
S6	100 microg	5 ng		15.00 MIF
S7 (neg)		5 ng	50 microg	15.00 MIF

Table 8. Different CKA2 protein concentrations.

S1-S3 showed that the MIF did not increase by using a higher amount of CKA2. This possibly suggested that the beads were already saturated with CKA2 by the amount of 25 microg of lysate. Thus, making clear that this was the amount of lysate to proceed with. The signal level of S4-S6 was in the same range as for the negative control. This demonstrated the high binding affinity of Thioredoxin to the beads and confirmed, again, the specific binding of CKA2 to the beads since the MIF decreased in the presence of this competitive protein.

6.2.4 Yeast interacting partners

Since the detection of CKA2 on the LiquiChip was shown by an increase in the MIF and the basic assay was established, we could go one step further by trying to implement the system on protein interacting partners. We chose CKA2×CKA1 to start with for its purpose as a positive control in other assays to follow.

6.2.4.1 Comparison of different extraction methods

Before further establishing the LiquiChip assay for the work with yeast we first wanted to compare two different extraction methods for the lysis of the yeast. So far, we had gathered our lysate by using glass beads, as to say by a mechanical method. Its advantage consisted of the fact that the extract contained as few unknown components as possible and the method was well established.

The other method of choice was Y-PER, a commercially available chemical. The initial trigger for the preference of the Y-PER extraction method compared to the glass beads was the very high quantity of its protein fraction: the end volume of lysate was at least 10 times higher while the protein concentration (mg/ml) was the same or even slightly higher. In addition the working procedure took only 1/3 of the time needed as when working with the glass beads extraction method. Thus making Y-PER an ideal tool for working in a high-throughput manner. However, it was clear that the method of choice would be the one giving a higher MIF, which ment producing less background and maintained the interactions between the proteins.

The two different extractions (Y-PER and glass beads) were produced out of the same cell fraction giving us a solid ground for comparing the data set. Since the difference in total protein concentration of the two extraction fractions was negligible, the tests could be performed with the same amount of lysate being used, making the comparison even more accurate.

The lysate was extracted from a diploid yeast line (an a-strain containing CKA2 was previously mated with an α-strain containing CKA1) so that the His-tagged CKA2 as well as the VSV-tagged CKA1 were present. While CKA2 would bind to the Ni-Beads as usual, it had additionally CKA1, its interacting partner, attached. Therefore, the primary antibody was not directed towards CKA2 anymore. The primary antibody was rabbit α-VSV-G binding to CKA1, detectable by the LiquiChip through the secondary antibody Alexa Fluor532 goat α-rabbit IgG. Since the previous results had shown that the Ni-Beads were already saturated by 25 micrograms of lysate, we chose to reduce the amount of lysate for the second samples to see if this would make any difference in the MIF. Additionally, Rem50$\times\alpha$ was used as a negative control. Although Rem50 could bind to the Ni-Beads with its His-tag, in this assay it could not be detected by the LiquiChip for the lack of a binding partner for the primary antibody.

1AB: rabbit α-VSV-G (50ng)

2AB: Alexa Fluor532 goat α-rabbit IgG (800ng)

Y-PER: protein extraction of CKA2×CKA1

Beads: protein extraction of CKA2×CKA1

Control: protein extraction of Rem50×α (Y-PER)

SAMPLE	Y-PER	BEADS	CONTROL	RESULT
S1	12.5 microg			148.00 MIF
S2	25.0 microg			147.00 MIF
S3		12.5 microg		354.00 MIF
S4		25.0 microg		242.00 MIF
S5			12.5 microg	137.00 MIF
S6			25.0 microg	109.00 MIF

Table 9. Comparing the Y-PER and the glass beads extractions.

The test showed that the lysate produced by the glass beads gave a higher signal. The MIF of the glass beads extracts was over 1.5 higher for the 25 micrograms samples (comparing S2 to S4) and almost 2.5 higher for the 12.5 micrograms fractions (comparing S1 to S3) than the extracts made with Y-PER. This suggested that the chemical treatment of the lysate using Y-PER had a stronger negative influence on the data outcome than the physical shearing performed by the glass beads, implicating that Y-PER's chemicals were either disturbing the protein interaction itself, the binding of the CKA2 to the beads, the annealing of the antibodies or the LiquiChip detection system. The results showed further, that an overall protein concentration of 12.5 micrograms contained enough of the tagged proteins for the LiquiChip to detect, while a higher concentration of extract started to influence the measurement in a negative way and lessened the MIF signal. Even though the MIF of S3 and S4 confirmed the ability of the LiquiChip to detect protein interactions by being 2.5 times higher than the negative control (comparing S3 to S5) its reliability was confirmed by performing another test using a negative control done by the glass beads extraction method.

6.2.4.2 Different yeast protein interaction partners

We wanted to expand our array of proteins being tested. Therefore, we implemented the LiquiChip technology on all our positive controls, as to say on the tetramer of the Protein Kinase II complex. To have a solid foundation for data comparison, the set-up had to fulfill the following guidelines:

Each sample had to have its own negative controls

Like this, the MIF produced by each specific protein interaction could be scaled more accurately, since the conditions of the negative controls were most similar. We included the proper VSV-tagged protein in the controls, which assured the binding of the antibodies to their target. However, without a bait to interact with, the signal given from the sample could be judged as unspecific background. One negative control consisted of an empty a-strain, making the binding of the Ni-Beads to a target protein impossible, while the other control included a His-tagged protein, which was not an interacting partner of the Protein Kinase II complex. Since in those set-ups the detection of the VSV-tagged protein should not be possible for the LiquiChip, by the lack of an interacting partner, the MIF reached by both negative controls should thus be on a similar level and significantly lower than the result of the positive sample.

Detection of interacting partners as bait×prey and prey×bait set-up

Since the LiquiChip technology was supposed to be a neutral detection method for protein interactions we had to ensure that its data represented the strength of an interaction and did not depend on the chosen set-up conditions, as to say on the fact which of the interacting partners was serving as the bait and which one as the prey.
This suggested that the tested proteins should be interchangeable within the pathway of detection (bait×prey or prey×bait) without a change in the output of the MIF. For this reason, we wanted to test the CKA2×CKA1 interaction in both possible set-ups. First in our already tested terms with a His-tagged CKA2 and second by letting the detection of the antibodies go over the binding on a VSV-tagged CKA2.

Reproducibility of the LiquiChip results

To prove the reliability of the LiquiChip technology, we double checked the data produced in both possible ways. First of all, the CKA2×CKA1 interaction was set-up

twice and then tested in the same throughput, ensuring the same conditions. Second, by repeating the experiment on the following days.

SAMPLE	HIS-TAG	VSV-TAG	RESULT
S1	CKA2	CKA1	310.25 MIF
S2 (neg)	empty a	CKA1	104.25 MIF
S3 (neg)	REM50	CKA1	92.00 MIF
S4	CKA2	CKA1	315.50 MIF
S5 (neg)	empty a	CKA1	90.00 MIF
S6	CKA1	CKA2	215.00 MIF
S7 (neg)	empty a	CKA2	89.50 MIF
S8 (neg)	ASF1	CKA2	90.50 MIF
S9	CKA1	CKA2	255.50 MIF
S10 (neg)	empty a	CKA2	99.25 MIF
S11	CKA2	CKB1	357.75 MIF
S12 (neg)	empty a	CKB1	108.25 MIF
S13 (neg)	REM50	CKB1	96.75 MIF
S14 (pos)	CKA1	CKB1	248.25 MIF

Table 10. Testing the positive controls in different set-ups.

Taken the average MIF of each VSV-tagged protein tested, we got the following results:

CKA1

The average MIF when together with CKA2 (S1+S4) was 312.88. Both negative controls were more than three times lower, namely 92 with REM50 as bait (S3) and 97.13 for the empty a-strain (S2+S4).

CKA2

The MIF of CKA2 interacting with the His-tagged CKA1 (S6+S9) was 235.25, whereas the MIF was only 90.5 for ASF1 (S8) and 94.38 for the other negative control (S7+10).

CKB1

It showed a slightly higher binding to CKA2 (S11) than to CKA1 (S14), both serving as positive controls, producing a MIF of 303. The MIF, when together with REM50 (S13) dropped to 96.75 and increased a bit for the empty a-strain to 108.25.

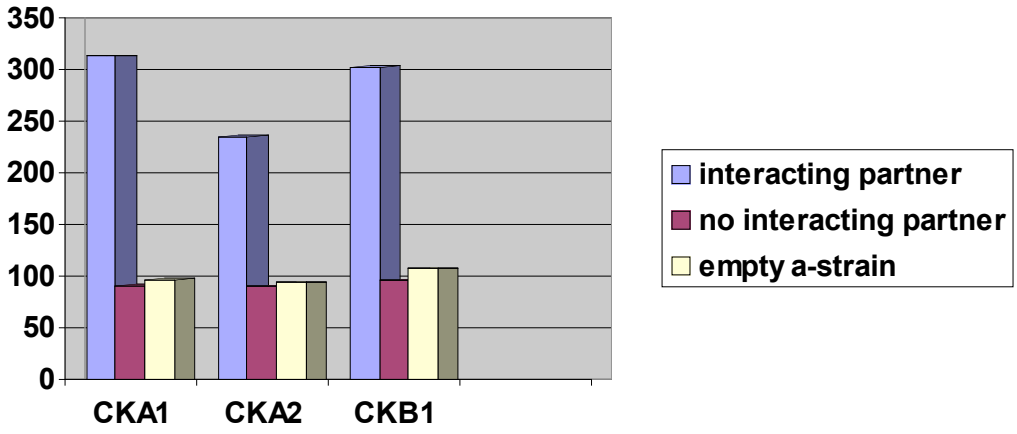

Figure 13. Comparing the MIF of the positive controls.

The results showed that the MIF for each protein interaction pair was around three times higher than the signal from their negative controls. This consistency of the MIF made clear that the protein interactions were measurable, and, therefore, detectable with the LiquiChip technology. Since the difference between the negative control containing a His-tagged protein and the one made out of an empty a-strain was constantly lower, the assumption that a MIF around 100 was caused by background, as to say by unspecific binding, was supported.

The consistency of the data gathered was tested by using the same set-ups (interacting partners) in different samples and running them at the same time and, in addition, repeating the whole set-up one day later. Since the parametrical variations of the MIF were very small the reliability of the LiquiChip method could be given as likely.

7 Discussion

7.1 Need for new proteomic approaches

The incredible speed in which new DNA sequencing data is created and, therefore, genomic science is progressing, calls for new and more efficient methods in the proteomic area. For the impact of this huge amount of newly gained information can only be evaluated if examined on the proteomic level. It is the proteins that hold the key to the overall understanding of the organization and the biochemical pathways of each living organism. Therefore, only by analyzing the function and behaviour of each protein within a cell the goal of genetics can be fulfilled. However, the complexity of the protein network within a cell makes clear that a protein's role and impact can only be understood completely, when examined within the context of its pathways of protein interactions.

There are two major difficulties which call for even more innovative and newer methods in the field of proteomics: first of all, the possible multifunctionality of a protein requires that multiple approaches using different conditions and interacting partners are performed in order to understand protein function. This reassures that the whole complexity of a protein's network can be obtained. Second, since each proteomic method brings a slight manipulation into the natural set-up, which can cause a falsification of the information being gained, the limitations of each technique must be realized and overcome or complemented by other additional techniques. If results can be confirmed by two or three separate proteomic techniques the quality of the data is unquestionably superior. Therefore, only by combining the output of as many independent methods as possible and scoring only interactions that are found in datasets of at least three methods an accuracy of around 80% can be reached (Von Mering *et al*., 2002). This makes clear that an accurate protein profile can only be obtained by examining its behaviour by different proteomic technologies using different molecular approaches as well as combining *in vivo* and *in vitro* studies.

7.1.1 Focus on genome stability

Each living cell is faced with the crucial task of keeping up its genome stability. This can be achieved by two different strategies. First of all, the prevention of DNA damage accomplished by the structure and organization of the cell, which are cell membrane, nucleus, cytoplasm, and nucleolus, building a physical barrier, and its components, such as golgi complex, lysosomes, vesicles, etc., destroying toxical elements. Second, by recognizing DNA damage and thus repairing it properly. However, the genome stability is not only put at risk by environmental influences but also by the cells own physiology. Throughout cell replication and recombination the cell has to reassure the equality of its DNA duplicate in order to guarantee genome stability for its daughter cells. This is achieved by multicomplex enzymatic processes including prove reading of the duplicated DNA, stopping the cell cycle by recognizing any mistakes and thus repairing the found mismatches before letting the cell enter into the next phase of the cycle.

Therefore, next to the cells physical components, it is the proteins involved in replication, recombination and repair, which have a tremendous impact in reassuring the functionality and the integrity of a cell and thus are responsible for the conservation of the intact genome by preventing possible mistakes by the creation of a new DNA fragment during the replication and recombination cycles or by randomly checking its integrity, being put at risk through ageing, environmental influences and toxical substances, as by the proteins involved in cell repair.

7.1.2 Working with *Saccaromyces cerevisiae*

Saccharomyces cerevisiae not only has been a powerful organism in traditional genetic studies (Botstein *et al*., 1997) but it also plays a leading role in the analyses of functional genomics (Kumar and Snyder, 2001). Due to its compact genome, its neglectable number of introns, the ease of its experimental manipulation and the possibility of homologous recombination, the baker's yeast is one of the most studied organisms in the proteomic community next to *Drosophila* and *C. elegans*. In addition, *S.cerevisiae* is an informative predictor of human gene function: there is an increased number of examples in which homology between yeast and human genes,

combined with an understanding of the function of the yeast genes, has allowed significant insight into the function of the human genes (Stagljar, 2002).

The key technologies on which protein studies are preformed in yeast are co-immunoprecipitation linked to mass spectrometry on the biochemical level and the YTH system on the genetic one. Nevertheless, the biggest task proteomics is still faced with today is establishing high-throughput methods which allow efficient screening of an entire protein interacting group and thus creating signalling network maps giving insight into the communication and functional pathways within a cell.

However, choosing yeast as model organism was not only influenced by the fact that it is an easy eukaryotic organism to work with in biochemical terms. Moreso the fact that a big yeast community already exists and, therefore, a decent amount of published validated data could be gathered, made *S. cerevisiae* an ideal target to test a new proteomic method and judge its impact by comparing the results. Apart from this, the fact that, after so many years, the function of thousands of yeast genes still remain obscure, due to the high degree of genetic redundancy in yeast (Tong *et al*., 2001), shows the urgent need for new methods in the elucidation of the yeast proteome.

7.2 Aim of the project

The aim of our work could be split up in two major goals. First of all, the generation of two new DNA libraries, containing endogenously tagged yeast ORFs of protein products being involved in cell repair, replication and recombination. We chose these protein groups for their tremendous impact they have in reassuring the functionality and the integrity of a cell. Additionally, we included proteins of yet unknown function hoping to illuminate their role in the protein interaction network. Second, the establishment of a novel high-throughput technique that could detect binary protein interactions with the help of the generated protein libraries. Considering the complexity of each biomolecular process, it is not enough to know the fundamental function of each protein. Also their exact signaling pathway, including their interacting partners, has to be analyzed. Only this can lead to a global understanding of the underlying basic question of how and why proteins interact.

For this reason, we wanted to create an *in vivo* assay study using the LiquiChip technology. Since the LiquiChip machine is able to determine the interaction status between two proteins, a bait and prey, we labeled the chosen open reading frames with exogenous protein tags.

7.2.1 Bait and prey array strain lines

We were able to tag a total amount of 927 ORFs all of them being involved in cell replication, repair and recombination, or of yet unknown function, thus creating a DNA library of 470 baits (6His tagged) and a prey library of 457 VSV tagged ORFs.
To ensure the reliability of the libraries, each newly created ORF had to undergo a distinct procedure containing several control steps reassuring the correct insertion of the tag into the genome as well as the ability to detect the expressed protein by the tag.

Of the 97 ORFs which were inviable as deletions, we successfully tagged 87 ORFs as both bait and preys, 8 as either bait or prey, and only 2 were refractory to tagging. This suggests that the small tags used in our arrays do not interrupt essential protein activities of the vast majority of the ORFs tagged.

G418 resistancy

The yeast strains were grown on G418 plates allowing only those lines to grow, which contained a Kan^r gene, obtained by the integration of the cassette into the genome.

Control PCR

A colony screen was performed to test the correct insertion of the tag. The PCR products were run on a gel to confirm the length of the bands (His: 554bp, VSV: 452bp). From 1144 sequenced colony PCR products, we obtained 470 sequence verified baits and 457 sequence verified preys. Therefore, 19% of the colony PCR products contained mutations within the ORF portion of the primer, the V5 epitope, the 6 histidines or the 3xVSV tags. These mutations could have arisen during primer synthesis, PCR amplification or homologous recombination.

Western blot

To test the correct expression and functionality of the tag, two different western blots were performed detecting different structures of the cassette. First, a mouse α-V5 antibody annealing to the V5 epitope in front of the specific tag. Second, a Penta-His antibody specifically for the bait, respectively a mouse α-VSV for the prey proteins.

Sequencing

Each ORF got sequenced to confirm genome stability and the exact insertion of the tag.

Pull downs

The binding of the His-tag to proper beads as well as to the VSV-tagged protein was tested by performing pull downs using nickelbeads.

7.2.2 LiquiChip technology

The major task consisted of combining our accomplished work with the set-up given by the LiquiChip machine in an efficient manner allowing high-throughput procedures as well as establishing a yeast based LiquiChip platform with reproducible and valid results. Therefore, our focus was set on the optimization of the LiquiChip assays as well as on the reliability of our results.

7.2.2.1 Set-up of the samples

The two proteins of interest first had to be brought into one cell line by mating the a-strain containing the bait with the α-strain expressing the prey. To select for diploids, expressing both bait and prey, the colonies were grown on medium, which lacked two essential aminoacids (Methionine and Leucine). Since the a-strain was able to produce Leucine but not Methionine, and the α-type was just the other way around, only the yeast which were diploid were able to synthesize both aminoacids and, therefore, survive. Additionally, cell lysis had to be performed to obtain the naturally expressed bait and prey tagged proteins. We chose two different extraction methods, glass beads, shearing the cell wall by mechanical action and thus influencing the cell

lysate on a neglectable level, and Y-PER, a strictly chemical approach fitting the expectations of a high-throughput technology by its speed and amount of output.
Although, both methods gave the same amount of protein concentration in the lysate, we were forced to proceed with the more time consuming method since comparing assays on the LiquiChip machine showed much higher signals for the glass beads extracts leading to the assumption that the chemicals contained in the Y-PER were influencing the signalling pathway of the LiquiChip technology.

Finally, the proper amount of yeast extract had to be evaluated. The difficulty consisted in the fact that we were working with samples knowing only the overall protein concentration but not the amount of the tagged proteins. Therefore, we first started competitive assays with a purified protein (Thioredoxin, 5ng) to get a reference for the amount of lysate needed. Later, we pinpointed the amount to 12.5mg lysate per assay by another competitive assay between the positive (CKA2×CKA1) and the negative (REM50×α) controls.

7.2.2.2 Control assays

To elaborate a solid baseground for the LiquiChip technology it was important to work with the same proteins throughout all the different tests. We chose CKA2, a member of the Protein Kinase II complex (α: CKA1/CKA2 and β: CKB1/CKB2) as a positive control for its well documented interactional bindings towards the other members of the tetramer. Additionally, REM50 served as a negative control, regulating the Ty1 transposition in the mitochondrial network and, therefore, highly unlikely to interact neither with the positive control nor with any of the proteins involved in cell replication, repair or recombination.
Although the binding of CKA2 to nickelbeads had been shown by pull downs, its binding to the beads of the LiquiChip technology had to be confirmed by competitive assays with Thioredoxin. Then, assays with CKA2 as a bait with different set-ups were performed to check the detectability of the LiquiChip on protein interactions.
For this reason, we tested the mating of CKA2×CKA1 with two negative controls, as to be CKA2×α and CKA2 haploid. Since the MIF of the actual interaction partners CKA2×CKA1 was over 3 times higher than the one of the two negative controls, we took this as a proof of the LiquiChip technology to detect binary yeast tagged protein

interaction partners. To confirm that the results were not dependant on the set-up of the prey and the bait, we redid the experiments by choosing CKA1 as bait and CKA2 as prey with the according negative controls and got similar results. The set-up then was also done taking CKB1 as a bait and CKA1 and CKA2 as interaction partners getting a MIF in the same range allowing to assume a given interaction.

To make sure that the results gathered by the LiquiChip technology were reproducible each sample was set-up twice and, therefore, tested two times in the same throughput and, additionally, the tests were redone on another day. The fact, that all of our results were reproducible proved the reliability of this newly elaborated detection system.

7.3 Conclusion

Although we could confirm the reproducibility of our results many more experiments need to be performed before the usefulness of the LiquiChip technology, as a reliable tool in the field of proteomics, is solidified.

First of all, protein pairs of known interaction different from the Protein Kinase II complex have to be tested, with their own negative controls, since although the 3 fold difference in MIF for the Casein Kinase II family was reproducible, it might be different for other protein pairs. Second, the MIF has to be classified leaving no assumption whether a result has to be judged as background or can be seen as a proof of protein interaction. Third, the negative controls have to be standardized, thus opening the possibility for a high-throughput approach. Forth, the sensitivity of the LiquiChip technology has to be investigated further. The minimal amount of protein concentration needed for the detection of the proteins of interest should be evaluated by testing interactions of low expressed proteins.

When all of these standardisations have been successful, the LiquiChip technology can be seen as a solid protein interaction detection method in the world of yeast proteomics and thus be applied to screen for the detection of novel interaction partners in *Saccaromyces cerevisiae*.

With this work, we set a baseground for further investigation into the world of proteomics in yeast. The yeast lines will give room for a variety of different proteomic approaches, such as co-localization experiments, immunoprecipitation assays, or the examination of protein expression levels from endogenous promotors and, therefore, can be an asset to the entire yeast community.

Since many of the yeast proteins involved in DNA processing have human homologues, which have been linked to human disease, further experiments could build a foundation towards understanding aberrant protein activity occurring in disease states with which a deeper insight into the development of pathological cell behaviour could be obtained opening up a wide range of medical approaches. Additionally, by linking known proteins to proteins of unknown function, putative roles could be assigned to the unknown proteins providing a better understanding of the process of genome stability.

In addition, the LiquiChip technology opens up a new detection system for protein interaction in yeast. The elaborated assay offers an ideal starting point for high-throughput screening. However, to increase efficiency several steps have to be further optimized: the mating of the yeast strains, the lysis of the diploids as well as the set-up for analysis. This could be achieved by automating these processes by using a replica robot.

By including the help of bioinformatics, protein interaction maps could be created. Given that approximately 40% of yeast proteins are conserved through eukaryotic evolution (Chervitz *et al*., 1998), a global yeast protein interaction map could provide a partical framework for understanding more complex proteomes. Significant data could, therefore, be translated to that of the rest of biology, as the set of proteins nominally encoded by the human genome is approximately 5-fold greater than the total number of yeast proteins, comprehensive analysis of the human proteome is feasible with current technology (Ho *et al*., 2002).

Table 11. Fact File.

ORF Name	Bait Number	Gene	Prey number	Haploid phenotype	Systematic Name
ABF1	113-5	ABF1	31-1	Inviable	YKL112w
ABF2	38-2	ABF2	21-6	Viable	YMR072w
APD1	52-6	APD1	52-6	Viable	YBR151w
ASF1	10-6	ASF1	15-6	Viable	YJL115w
ASM4	07-5	ASM4	99-4	Viable	YDL088c
BUB1	21-1	BUB1	20-5	Viable	YGR188c
CAC2	27-6	CAC2	36-7	Viable	YML102w
CCE1	33-8	CCE1	17-7	Viable	YKL011c
CCL1	15-2	CCL1	17-4	Inviable	YPR025c
CDC1	31-1	CDC1	22-5	Inviable	YDR182w
CDC13	111-5	CDC13	99-7	Inviable	YDL220c
CDC14	121-8	CDC14	38-6	Inviable	YFR028c
CDC2	12-3	CDC2	29-1	Inviable	YDL102w
CDC28	99-3	CDC28	35-1	Inviable	YBR160w
CDC34	33-2	CDC34	38-4	Inviable	YDR054c
CDC4	92-5	CDC4		Inviable	YFL009w
CDC40	27-7	CDC40	29-5	Viable	YDR364c
CDC45	49-6	CDC45	114-2	Inviable	YLR103c
CDC46	28-1	CDC46	30-1	Inviable	YLR274w
CDC47	28-6	CDC47	98-3	Inviable	YBR202w
CDC5	32-6	CDC5	108-6	Inviable	YMR001c
CDC54	32-3	CDC54	41-7	Inviable	YPR019w
CDC6	106-7	CDC6		Inviable	YJL194w
CDC7	40-5	CDC7	39-4	Inviable	YDL071w
CDC9	11-3	CDC9	13-4	Inviable	YDL164c
CHK1	112-3	CHK1	109-6	Viable	YBR274w
CKA 2	03-2	CKA2	05-1	Viable	YOR061w
CKA1	09-7	CKA1	02-2	Viable	YIL035c
CKB 2	03-5	CKB2	02-7	Viable	YOR039w
CKB1	14-6	CKB1	02-5	Viable	YGL019w
CLB5	100-7	CLB5	115-3	Viable	YPR120c
CLB6	30-7	CLB6	34-3	Viable	YGR109c
CLN2	30-8	CLN2	98-6	Viable	YPL256c
CLN3	31-4	CLN3	101-3	Viable	YAL040c
CST6	28-7	CST6	31-8	Viable	YIL036w
CST9	35-2	CST9	34-6	Viable	YLR394w
CTF4	09-3	CTF4	06-6	Viable	YPR135w
DBF4	121-7	DBF4	39-1	Inviable	YDR052c
DBP2		DBP2		Inviable	YNL112w
DBP3	50-4	DBP3	43-6	Viable	YGL078c
DDC1	19-1	DDC1	26-6	Viable	YPL194w
DMC1	06-9	DMC1	02-9	Viable	YER179w
DNA2	02-9	DNA2	03-6	Inviable	YHR164c
DNL4	08-6	DNL4	24-6	Viable	YOR005c

DPB11	32-8	DPB11	38-2	Inviable	YJL090c
DPB3	52-7	DPB3	61-3	Viable	YBR278w
DST1	37-2	DST1	35-7	Viable	YGL043w
DUN1	10-7	DUN1	26-5	Viable	YDL101c
ECM32	08-1	ECM32	114-6	Viable	YER176w
ECO1	31-6	ECO1	40-8	Inviable	YFR027w
ELG1	112-1	ELG1	110-1	Viable	YOR144c
EST2	108-2	EST2		Viable	YLR318w
EXO1	123-5	EXO1	112-8	Viable	YOR033c
FOB1	51-2	FOB1	18-3	Viable	YDR110w
FUN30	17-4	FUN30	112-6	Viable	YAL019w
GIS1	37-8	GIS1	42-8	Viable	YDR096w
HMI1	50-2	HMI1	43-8	Viable	YOL095c
HOP1	39-6	HOP1	27-7	Viable	YIL072w
HPR1	112-7	HPR1	115-4	Viable	YDR138w
HPR5	108-4	HPR5	103-5	Viable	YJL092w
HRR25	107-6	HRR25	110-8	Inviable	YPL204w
HSM3	34-6	HSM3	32-1	Viable	YBR272c
HSP12	58-2	HSP12	97-5	Viable	YFL014w
HUG1	119-6	HUG1	104-4	Viable	YML058w-a
HYS2	11-8	HYS2	25-1	Inviable	YJR006w
IMP2	14-4	IMP2	14-6	Viable	YMR035W
KEM1	24-7	KEM1	32-3	Viable	YGL173c
KIN28	109-4	KIN28	105-7	Inviable	YDL108w
LCD1	23-4	LCD1	21-3	Inviable	YDR499w
LIF1	08-7	LIF1	14-3	Viable	YGL090w
MCM2	34-8	MCM2	40-6	inviable	YBL023c
MCM3	49-8	MCM3	39-3	inviable	YEL032w
MCM6	32-4	MCM6	45-6	inviable	YGL201c
MEC1	42-3	MEC1	29-1	inviable	YBR136w
MEC3	12-8	MEC3	114-5	viable	YLR288c
MEI5	34-4	MEI5	114-8	viable	YPL121c
MEK1	36-2	MEK1	36-2	viable	YOR351c
MER1	50-7	MER1	94-1	viable	YNL210w
MER3	105-5	MER3	102-1	viable	YGL251c
MET18	13-4	MET18	14-4	viable	YIL128w
MGM101	14-2	MGM101	14-5	viable	YJR144w
MGT1	20-1	MGT1	28-3	viable	YDL200c
MHR1	21-3	MHR1	18-6	viable	YDR296w
MIP1	122-6	MIP1	114-4	viable	YOR330c
MLH1	18-4	MLH1	03-2	viable	YMR167w
MLH2	04-5	MLH2	36-5	viable	YLR035c
MLH3	17-8	MLH3	30-5	viable	YPL164c
MLP1	20-5	MLP1	114-7	viable	YKR095w
MMS1	10-1	MMS1	15-1	viable	YPR164w
MMS2	122-1	MMS2	107-5	viable	YGL087c
MMS21	08-8	MMS21	25-7	inviable	YEL019c
MMS22	10-2	MMS22	15-2	viable	YLR320w
MMS4	21-7	MMS4	24-4	viable	YBR098w
MPH1	04-1	MPH1	06-5	viable	YIR002c
MRC1	111-7	MRC1	108-5	viable	YCL061c
MRE11	16-2	MRE11	17-6	viable	YMR224c

MSH1	51-4	MSH1	21-5	viable	YHR120w
MSH2	03-8	MSH2	36-3	viable	YOL090w
MSH3	92-6	MSH3	16-8	viable	YCR092c
MSH4	23-2	MSH4	20-6	viable	YFL003c
MSH5	25-2	MSH5	21-2	viable	YDL154w
MSH6	51-6	MSH6	17-2	viable	YDR097c
MSI1	29-5	MSI1	33-7	viable	YIR009w
MTF1	36-1	MTF1	42-7	viable	YMR228w
MUS81	13-6	MUS81	14-2	viable	YDR386w
NAM8	20-7	NAM8	27-3	viable	YHR086w
NEJ1	111-1	NEJ1	108-4	viable	YLR265c
NHP6A	118-3	NHP6A	113-1	viable	YPR052c
NHP6B	113-1	NHP6B	109-2	viable	YBR089c-a
NTG1	09-1	NTG1	11-5	viable	YAL015c
NTG2	112-6	NTG2	13-2	viable	YOL043c
ORC1	38-4	ORC1	54-6	inviable	YML065w
ORC2	43-5	ORC2	40-3	inviable	YBR060c
ORC3	43-4	ORC3	43-7	inviable	YLL004w
ORC4	42-1	ORC4	111-4	inviable	YPR162c
ORC5	34-2	ORC5	43-3	inviable	YNL261w
ORC6	43-1	ORC6	40-4	inviable	YHR118c
PAF1		PAF1		viable	YBR279w
PAT1	26-2	PAT1	31-2	viable	YCR077c
PES4	18-3	PES4	12-6	viable	YFR023w
PGD1	19-5	PGD1	17-8	viable	YGL025c
PHB1	114-5	PHB1	39-6	viable	YGR132c
PHB2	42-5	PHB2	39-8	viable	YGR231c
PHR1	07-8	PHR1	07-8	viable	YOR386w
PIF1	18-6	PIF1A	09-5	viable	YML061c
PMS1	22-8	PMS1	21-8	viable	YNL082w
POB3	121-3	POB3	31-6	inviable	YML069w
POL1	38-7	POL1	35-3	inviable	YNL102w
POL12	53-1	POL12	56-3	inviable	YBL035c
POL2	12-4	POL2	12-8	inviable	YNL262w
POL30	02-1	POL30	108-2	inviable	YBR088c
POL32	15-4	POL32	26-3	viable	YJR043c
POL4	106-3	POL4	28-7	viable	YCR014c
PRI1	41-7	PRI1	48-8	inviable	YIR008c
PRI2	92-3	PRI2	37-7	inviable	YKL045w
PSO2	11-6	PSO2	11-8	viable	YMR137c
RAD1	7-7	RAD1	116-2	viable	YPL022w
RAD10	18-1	RAD10	12-2	viable	YML095c
RAD14		RAD14		viable	YMR201c
RAD16	19-2	RAD16	116-3	viable	YBR114w
RAD17	54-3	RAD17	65-4	viable	YOR368w
RAD18	14-8	RAD18	15-4	viable	YCR066w
RAD2	04-7	RAD2	06-3	viable	YGR258c
RAD23	17-7	RAD23	112-7	viable	YEL037c
RAD24	100-1	RAD24	40-7	viable	YER173w
RAD26	03-7	RAD26	116-6	viable	YJR035w
RAD27	01-2	RAD27	48-4	viable	YKL113c
RAD28	04-4	RAD28	115-5	viable	YDR030c
RAD3	04-6	RAD3	07-1	inviable	YER171w

RAD30	54-4	RAD30	13-3	viable	YDR419w
RAD4	04-8	RAD4	07-2	viable	YER162c
RAD5	08-3	RAD5	08-6	viable	YLR032w
RAD50	101-6	RAD50	103-7	viable	YNL250w
RAD51	02-3	RAD51	05-4	viable	YER095w
RAD52	108-1	RAD52	07-9	viable	YML032c
RAD53	19-3	RAD53	23-7	inviable	YPL153c
RAD54	20-3	RAD54	111-3	viable	YGL163c
RAD55	09-2	RAD55	11-6	viable	YDR076w
RAD57	98-7	RAD57	16-6	viable	YDR004w
RAD59	36-8	RAD59	32-5	viable	YDL059c
RAD6	05-5	RAD6	07-5	viable	YGL058w
RAD7	118-1	RAD7	28-1	viable	YJR052w
RAD9	97-7	RAD9	16-4	viable	YDR217c
RDH54	118-2	RDH54	102-7	viable	YBR073w
REC102	41-2	REC102	96-6	viable	YLR329w
REC104	19-7	REC104	18-1	viable	YHR157w
REC107	24-4	REC107	29-8	viable	YJR021c
RED1	24-8	RED1	22-2	viable	YLR263w
REM50	05-2	REM50	08-3	viable	YLL002w
REV1	52-4	REV1	115-7	viable	YOR346w
REV3	05-4	REV3	08-4	viable	YPL167c
REV7	11-5	REV7	13-5	viable	YIL139c
RFA1	123-2	RFA1	23-6	inviable	YAR007c
RFA2	118-5	RFA2	99-8	inviable	YNL312w
RFA3	101-7	RFA3	93-8	inviable	YJL173c
RFC1	39-2	RFC1	45-5	inviable	YOR217w
RFC2	38-6	RFC2	35-2	inviable	YJR068w
RFC3	44-4	RFC3	94-8	inviable	YNL290w
RFC4	43-8	RFC4	88-2	inviable	YOL094c
RFC5	40-3	RFC5	87-7	inviable	YBR087w
RHC18		RHC18	95-4	inviable	YLR383w
RIF2	02-8	RIF2	05-2	viable	YLR453c
RLF2	01-4	RLF2	49-7	viable	YPR018w
RNR3	11-2	RNR3	115-8	viable	YIL066c
RNR4	16-4	RNR4	24-1	viable	YGR180c
RPA1	01-8	RPA1	03-4	inviable	YAR007c
RRD1	04-2	RRD1	06-4	viable	YIL153w
RRM3		RRM3		viable	YHR031c
RRP40	117-7	RRP40	30-7	inviable	YOL142w
RRP41	33-4	RRP41	96-1	inviable	YFL036w
RTS2	36-4	RTS2	33-6	inviable	YOR077w
RTT107	09-5	RTT107	14-8	viable	YHR154w
SAE2	21-6	SAE2	16-3	viable	YGL175c
SEM1	109-2	SEM1	110-3	viable	YDR363w-a
SGS1	107-5	SGS1	50-2	viable	YMR190c
SIR2	17-3	SIR2	19-6	viable	YDL042c
SIR3	22-3	SIR3	20-4	viable	YLR442c
SIR4	16-5	SIR4	19-3	viable	YDR227w
SKI8	01-6	SKI8	19-2	viable	YGL213c
SLD2	35-5	SLD2	70-2	inviable	YKL108w
SLX1	06-2	SLX1	05-5	viable	YBR228w

SLX4	112-5	SLX4	101-6	viable	YLR135w
SMC1	123-3	SMC1	100-4	inviable	YFL008w
SMC5	50-5	SMC5	43-4	inviable	YOL034w
SML1	24-5	SML1	32-7	viable	YML058w
SOH1	53-8	SOH1	53-3	viable	YGL127c
SOY1	57-1	SOY1	53-4	viable	YBR194w
SPO11	22-2	SPO11	24-5	viable	YHL022c
SPO7	57-4	SPO7	53-8	viable	YAL009w
SPT4	26-4	SPT4	31-7	viable	YGR063c
SPT6	26-6	SPT6	90-4	inviable	YGR116w
SSL1	97-4	SSL1	16-2	inviable	YLR005w
SSL2	23-3	SSL2	06-2	inviable	YIL143c
SSO1	33-6	SSO1	42-2	viable	YPL232w
STN1	111-3	STN1		inviable	YDR082w
SWC1	57-2	SWC1	56-1	viable	YAL011w
SWI6	113-7	SWI6	31-5	viable	YLR182w
TBS1		TBS1		viable	YBR150c
TDP1	51-8	TDP1	54-4	inviable	YBR223c
TEL1	53-7	TEL1	118-8	viable	YBL088c
TEN1	122-2	TEN1	114-3	inviable	YLR010c
TFB1	17-1	TFB1	118-6	inviable	YDR311w
TFB2	116-4	TFB2	33-8	inviable	YPL122c
TFB3	15-7	TFB3	20-2	inviable	YDR460w
TFB4	115-6	TFB4	08-8	inviable	YPR056w
THI4	14-1	THI4	37-4	viable	YGR144w
TOF1	25-8	TOF1	34-5	viable	YNL273w
TOP1	17-5	TOP1	91-1	viable	YOL006c
TOP2	123-4	TOP2	117-2	inviable	YNL088w
TOP3	02-5	TOP3	03-3	viable	YLR234w
UNG1	113-4	UNG1	33-1	viable	YML021c
WBP1		WBP1			YEL002c
WSS1	21-9	WSS1	27-1	viable	YHR134w
XRS2	16-3	XRS2	26-7	viable	YDR369c
YAL027w	44-8	YAL027w	55-6	viable	
YAL061w	94-4	YAL061w	85-7	viable	
YBL019w	08-4	YBL019w	10-4	viable	
YBL028c	43-7	YBL028c	56-5	viable	
YBL029w	112-8	YBL029w	119-3	viable	
YBL036c	70-3	YBL036c	69-7	viable	
YBL046w	96-1	YBL046w	90-7	viable	
YBL054w	58-4	YBL054w	52-2	viable	
YBL059c-a	123-6	YBL059c-a	112-3		
YBR030w	66-8	YBR030w	84-1	viable	
YBR085c-a	119-1	YBR085c-a	113-6		
YBR090c	117-2	YBR090c	107-2	viable	
YBR094w	05-8	YBR094w	05-7	viable	
YBR111c		YBR111c		viable	
YBR197c	72-6	YBR197c	60-4	viable	
YBR239c	78-1	YBR239c	83-6	viable	
YBR242w	61-7	YBR242w	58-5	viable	
YBR281c	66-4	YBR281c	95-1	viable	
YCL026c-b	77-3	YCL026c-b	65-5		
YCR016w	59-5	YCR016w	50-5	viable	
YCR018c-a	118-8	YCR018c-a			

YCR020w-b	120-8	YCR020w-b	100-6	viable
YCR051w	82-6	YCR051w	103-3	viable
YCR060w	98-3	YCR060w	117-1	viable
YCR082w	73-6	YCR082w	67-5	viable
YCR090c	76-1	YCR090c	112-2	viable
YDL036c	66-1	YDL036c	88-4	viable
YDL053c	102-7	YDL053c	79-8	viable
YDL063c	109-5	YDL063c	105-3	viable
YDL085c-a	121-5	YDL085c-a	107-7	
YDL110c	79-3	YDL110c	69-5	viable
YDL124w	101-4	YDL124w	80-2	viable
YDL129w	44-6	YDL129w	49-6	viable
YDL144c	44-3	YDL144c	44-5	viable
YDL152w	75-6	YDL152w	92-8	inviable
YDL156w	122-3	YDL156w	116-8	viable
YDL233w	49-5	YDL233w	48-7	viable
YDR020c	71-8	YDR020c	69-7	viable
YDR031w	117-8	YDR031w	61-6	viable
YDR051c	94-5	YDR051c	102-8	viable
YDR061w	06-5	YDR061w	117-6	viable
YDR063w	77-8	YDR063w	82-2	viable
YDR079c-a	118-4	YDR079c-a	109-7	
YDR111c	105-8	YDR111c	79-4	viable
YDR132c	44-5	YDR132c	47-1	viable
YDR193w	56-3	YDR193w	56-7	viable
YDR288w	56-5	YDR288w	54-2	inviable
YDR291w	85-1	YDR291w	75-3	viable
YDR314c	90-6	YDR314c	80-8	viable
YDR330w	61-1	YDR330w	65-1	viable
YDR365c	89-6	YDR365c	102-3	inviable
YDR412w	89-4	YDR412w	89-7	inviable
YDR514c	73-2	YDR514c	59-4	viable
YDR520c	79-5	YDR520c	81-5	viable
YDR531w	87-4	YDR531w	96-4	inviable
YDR533c	60-7	YDR533c	112-4	viable
YDR540c	81-5	YDR540c	82-8	viable
YEL008w	96-8	YEL008w	76-2	viable
YEL047c	90-8	YEL047c	78-3	viable
YER030w	64-7	YER030w	113-7	viable
YER034w	85-3	YER034w	71-6	viable
YER049w	63-5	YER049w	113-8	viable
YER064c	44-1	YER064c	46-7	viable
YER067w	89-1	YER067w	78-2	viable
YER079w	55-6	YER079w	51-6	viable
YER084w	91-4	YER084w	78-5	viable
YER134c	68-6	YER134c	64-1	viable
YER142c	06-4	YER142c	09-9	viable
YER152c		YER152c		viable
YER156c	86-4	YER156c	45-2	viable
YER163c	85-8	YER163c	44-6	viable
YFL044c	110-4	YFL044c	93-5	viable
YFL049w	64-6	YFL049w	60-6	viable
YFR038w	122-8	YFR038w	37-1	viable

YFR046c		YFR046c		viable
YGL082w	104-4	YGL082w	48-5	viable
YGL101w	74-6	YGL101w	117-8	viable
YGL157w	65-3	YGL157w	58-3	viable
YGL220w	104-5	YGL220w	79-2	viable
YGL232w	119-3	YGL232w	117-5	viable
YGL250w	65-7	YGL250w	90-2	viable
YGR017w	83-4	YGR017w	74-5	viable
YGR024c	77-1	YGR024c	66-4	inviable
YGR042w	90-2	YGR042w	77-6	viable
YGR043c	88-2	YGR043c	76-6	viable
YGR052w	80-8	YGR052w	68-5	viable
YGR058w	110-2	YGR058w	105-5	viable
YGR071c	89-3	YGR071c	77-8	viable
YGR081c	60-3	YGR081c	115-2	viable
YGR093w	75-2	YGR093w	64-3	viable
YGR111w	96-2	YGR111w	118-3	viable
YGR126w	45-3	YGR126w	44-8	viable
YGR151c	55-4	YGR151c	51-3	viable
YGR203w	49-3	YGR203w	44-3	viable
YGR205w	69-3	YGR205w	69-1	viable
YGR219w	95-6	YGR219w	96-3	viable
YGR251w	75-4	YGR251w	68-1	inviable
YGR269w	49-4	YGR269w	45-1	viable
YGR272c	90-1	YGR272c	116-7	viable
YHR009c	114-1	YHR009c	83-3	viable
YHR034c	59-3	YHR034c	87-3	viable
YHR087w	71-2	YHR087w	63-3	viable
YHR097c		YHR097c	101-1	viable
YHR121w	101-1	YHR121w	72-3	viable
YHR132w-a	87-5	YHR132w-a	91-4	viable
YIL019w	59-7	YIL019w	55-4	inviable
YIL083c	60-5	YIL083c	87-5	inviable
YIL091c	91-5	YIL091c	79-6	inviable
YIL092w	81-3	YIL092w	70-4	viable
YIL096c	54-2	YIL096c	56-2	viable
YIL110w	103-1	YIL110w	78-7	viable
YJL010c	98-5	YJL010c	75-7	inviable
YJL055w	62-5	YJL055w	59-8	viable
YJL065c	44-7	YJL065c	45-8	viable
YJL122w	86-7	YJL122w	50-4	viable
YJL162c	63-7	YJL162c	60-8	viable
YJL169w	62-4	YJL169w	82-6	viable
YJR008w	72-1	YJR008w	71-1	viable
YJR056c	45-2	YJR056c	48-1	viable
YJR070c	45-4	YJR070c	65-2	viable
YJR096w	60-1	YJR096w	97-2	viable
YJR097w	97-6	YJR097w	103-2	viable
YJR119c	99-2	YJR119c	72-6	viable
YJR146w	104-2	YJR146w	101-4	viable
YKL069w	95-5	YKL069w	86-5	viable
YKL086w	121-4	YKL086w		viable
YKL091c	70-7	YKL091c	62-7	viable
YKL102c	54-8	YKL102c	50-8	viable

YKL114c	53-3	YKL114c	10-2	viable	
YKL145w	07-1	YKL145w	09-7	inviable	
YKL169c	70-5	YKL169c	91-6	viable	
YKR018c	68-7	YKR018c	63-1	viable	
YKR022c	66-5	YKR022c	57-8	inviable	
YKR041w	93-1	YKR041w	86-7	viable	
YKR043c	80-2	YKR043c	69-3	viable	
YKR064w	83-6	YKR064w	75-2	viable	
YKR074w	58-6	YKR074w	47-8	viable	
YKR075c	73-4	YKR075c	60-2	viable	
YKR077w	55-2	YKR077w	51-1	viable	
YKR079c	72-3	YKR079c	60-3	inviable	
YKR096w	99-7	YKR096w	90-5	viable	
YKR103w	71-5	YKR103w	111-6	viable	
YKU70	13-2	YKU70	13-8	viable	YMR284W
YKU80	08-5	YKU80	10-7	viable	YMR106C
YLL034c	59-4	YLL034c	112-1	inviable	
YLR003c	103-2	YLR003c	93-7	viable	
YLR016c	76-8	YLR016c	62-4	viable	
YLR022c	67-6	YLR022c	61-8	inviable	
YLR108c	80-1 (2)	YLR108c	82-1	viable	
YLR118c	93-8	YLR118c	85-4	viable	
YLR132c	62-6	YLR132c	83-4	inviable	
YLR145w	100-3	YLR145w	73-5	inviable	
YLR179c	67-3	YLR179c	91-8	viable	
YLR218c	95-1	YLR218c	86-2	viable	
YLR254c	103-3	YLR254c	74-2	viable	
YLR262c-a	121-6	YLR262c-a	113-5	viable	
YLR271w	77-6	YLR271w	66-6	viable	
YLR278c	88-6	YLR278c	77-1	viable	
YLR301w	94-2	YLR301w	70-5	viable	
YLR327c	110-5	YLR327c	100-7	viable	
YLR352w	122-5	YLR352w	113-3	viable	
YLR363w-a	122-7	YLR363w-a	107-4	no data	
YLR385c	73-8	YLR385c	66-7	viable	
YLR424w	96-7	YLR424w	89-4	inviable	
YLR455w	109-8	YLR455w	84-8	viable	
YML011c	68-1	YML011c	58-1	viable	
YML053c	45-7	YML053c	55-8	viable	
YML060c	07-3	YML060w	25-3	viable	
YML079w	72-8	YML079w	57-4	viable	
YML081w	74-4	YML081w	63-7	viable	
YML082w	96-4	YML082w	89-2	viable	
YML108w	54-6	YML108w	50-7	viable	
YMR002w	82-3	YMR002w	89-8	viable	
YMR009w	84-7	YMR009w	94-3	viable	
YMR030w	99-5	YMR030w	75-1	viable	
YMR067c	91-3	YMR067c	80-4	viable	
YMR074c	83-8	YMR074c	71-5	viable	
YMR099c	82-8	YMR099c	74-7	viable	
YMR111c	80-6	YMR111c	70-1	viable	
YMR114c	104-8	YMR114c	88-7	viable	
YMR144w	78-7	YMR144w	95-5	viable	
YMR178w	87-1	YMR178w	48-2	viable	

YMR226c	91-1	YMR226c	78-8	viable
YMR244c-a	78-3	YMR244c-a	118-1	viable
YMR258c	59-1	YMR258c	55-2	viable
YMR278w	80-5	YMR278w	68-3	viable
YMR291w	47-5	YMR291w	49-5	viable
YMR310c	66-3	YMR310c	103-8	viable
YMR315w	76-5	YMR315w	67-1	viable
YNL010w	118-3	YNL010w	62-1	viable
YNL022c	81-7	YNL022c	76-1	viable
YNL035c	88-4	YNL035c	77-2	viable
YNL045w	91-7	YNL045w	81-2	viable
YNL108c	48-7	YNL108c	49-4	viable
YNL123w	82-1	YNL123w	73-2	viable
YNL134c	79-2	YNL134c	70-6	viable
YNL136w		YNL136w		viable
YNL143c	63-8	YNL143c	59-1	viable
YNL155w	47-7	YNL155w	117-7	viable
YNL157w	48-1	YNL157w	93-3	viable
YNL162w-a	83-3	YNL162w-a	73-1	no data
YNL171c	57-6	YNL171c	95-3	viable
YNL224c	55-8	YNL224c	51-7	viable
YNL254c	101-8	YNL254c	80-6	viable
YNL274c	78-5	YNL274c	68-7	viable
YNL313c	85-7	YNL313c	71-7	inviable
YNR009w	56-7	YNR009w	59-6	viable
YNR024w	48-3	YNR024w	47-3	viable
YNR046w	116-6	YNR046w	107-1	inviable
YOL032w	74-8	YOL032w	115-1	viable
YOL057w	87-8	YOL057w	91-5	viable
YOL125w	92-7	YOL125w	91-3	viable
YOL134c	79-7	YOL134c	104-6	inviable
YOR006c	87-2	YOR006c	55-5	viable
YOR051c	120-5	YOR051c	81-4	viable
YOR052c	58-7	YOR052c	116-5	viable
YOR060c	67-5	YOR060c	112-5	inviable
YOR062c	98-1	YOR062c	72-4	viable
YOR073w	94-6	YOR073w	85-8	viable
YOR131c	48-6	YOR131c	70-8	viable
YOR138c	84-4	YOR138c	76-4	viable
YOR179c	58-8	YOR179c	81-6	viable
YOR252w	64-1	YOR252w	61-2	viable
YOR283w	114-3	YOR283w	94-6	viable
YOR289w	82-5	YOR289w	73-7	viable
YOR302w	115-8	YOR302w	107-8	viable
YOR315w	68-3	YOR315w	71-2	viable
YOR342c	56-2	YOR342c	92-3	viable
YOR352w	90-4	YOR352w	77-7	viable
YPL014w	46-5	YPL014w	47-6	viable
YPL047w	120-3	YPL047w	100-5	viable
YPL068c	122-4	YPL068c	85-5	viable
YPL071c	94-3	YPL071c	85-6	viable
YPL110c		YPL110c	101-7	viable
YPL146c	64-3	YPL146c	59-2	inviable

YPL166w	93-4	YPL166w	84-5	viable	
YPL208w	46-7	YPL208w	56-6	viable	
YPL245w	47-1	YPL245w	116-4	viable	
YPL247c	87-7	YPL247c	76-8	Viable	
YPL260w		YPL260w	84-4	Viable	
YPR022c	85-5	YPR022c	111-7	Viable	
YPR045c	84-5	YPR045c	71-8	Viable	
YPR116w	05-9	YPR116w	106-1	Viable	
YPR118w	105-7	YPR118w	117-3	Viable	
YPR127w	84-3	YPR127w	73-4	Viable	
YPR143w	84-1	YPR143w	75-5	Inviable	
YPR152c	95-2	YPR152c	86-4	Viable	
YPR172w	62-8	YPR172w	46-4	Viable	
YRF1-1	27-2	YRF1-1	33-3	no data	YDR545w
YRF1-2	123-8	YRF1-2	114-1	no data	YER190w
YRF1-4	37-4	YRF1-4	35-6	no data	YLR466w
YSA1	108-5	YSA1	115-6	Viable	YBR111c
ZIP2	36-6	ZIP2	97-1	Viable	YGL249w
ZTA1	63-2	ZTA1	51-8	Viable	YBR046c

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